



ENZYME

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The present invention relates to enzymes, to polynucleotides encoding enzymes and to uses of enzymes and polynucleotides.

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Protein kinase B (PKB) [1] or RAC protein kinase [2] is the cellular homologue of a viral oncogene v-Akt [3] and has therefore also been termed c-Akt. The current interest in PKB stems firstly from the discovery that it is activated rapidly in response to insulin and growth factors and that the activation is prevented by inhibitors of phosphoinositide (PI) 3-kinase [4-6]; secondly, from the finding that PKB isoforms are overexpressed in a significant percentage of ovarian, pancreatic [7, 8] and breast cancer cells [2].

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PKB appears to mediate the insulin-induced inhibition of glycogen synthase kinase-3 (GSK3) in L6 myotubes which is thought to underlie, at least in part, the insulin-induced dephosphorylation and activation of glycogen synthase [9] and protein synthesis initiation factor eIF2 [10] that contribute to the stimulation of glycogen and protein synthesis by insulin. However PK2B is likely to have other physiological substrates and, in transfection based experiments, has been shown to activate pT0 \$6 kinase [5], to stimulate the translocation of the glucose transporter GLUT4 to the plasma membrane and enhance glucose uptake into 3T3-L1 adipocytes [11], and to mediate the IGF1-induced survival of neurones [12] and fibroblasts [13] against apoptosis.

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A critical question concerns the mechanism by which PI 3-kinase triggers the activation of PKB. The activation of PKB is accompanied by its phosphorylation [5, 14] and we recently showed that activation by insulin or IGF1 resulted from its phosphorylation at Thr-308 and Ser-473 [15].

Moreover, the insulin or IGF1 induced phosphorylation of both residues was abolished by wortmannin, an inhibitor of PI 3-kinase [15]. We believe that the protein kinases which phosphorylate PKB at Thr-308 and Ser-473 might themselves be activated by phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P3), the product of the PI 3-kinase reaction. In the work presented here, we demonstrate that this is indeed the case, and we describe the purification and characterisation of a 3-phosphoinositide-dependent protein kinase (PDK1) which activates PKB and we disclose polynucleotides which encode PDK1 and uses for PDK1 and said polynucleotides.

A first aspect of the invention provides a substantially pure 3-phosphoinositide dependent protein kinase that phosphorylates and activates protein kinase B α .

By "substantially pure" we mean that the 3-phosphoinositide-dependent protein kinase is substantially free of other proteins. Thus, we include any composition that includes at least 30% of the protein content by weight as the said 3-phosphoinositide-dependent protein kinase, preferably at least 50%, more preferably at least 70%, still more preferably at least 90% and most preferably at least 95% of the protein content is the said 3-phosphoinositide-dependent protein kinase.

Thus, the invention also includes compositions comprising the 3-phosphoinositide-dependent protein kinase and a contaminant wherein the contaminant comprises less than 70% of the composition by weight, preferably less than 50% of the composition, more preferably less than 30% of the composition, still more preferably less than 10% of the composition and most preferably less than 5% of the composition by weight.

The invention also includes the substantially pure said 3-phosphoinositide-dependent protein kinase when combined with other components *ex vivo*, said other components not being all of the components found in the cell in which said protein kinase is found.

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It is preferred that the substantially pure 3-phosphoinositide-dependent protein kinase is a substantially pure phosphatidyl-3,4-5-trisphosphate-dependent protein kinase or a substantially pure phosphatidyl-3,4-bisphosphate-dependent protein kinase.

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By "phosphorylates protein kinase B α " we include the meaning that the 3-phosphoinositide-dependent protein kinase is able to transfer a phosphate group from ATP to an acceptor group of protein kinase B α . Preferably, the acceptor group is Thr-308.

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By "protein kinase B α " we include any protein kinase B α or any suitable derivative or fragment thereof or fusion of protein kinase B α or derivative, or fragment thereof. For example, it is particularly preferred that the protein kinase B α is a fusion between glutathione-S-transferase and protein kinase B α as described in Example 1 (GST-PKB α ; see also reference 27).

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It is preferred that the PKB α is a human PKB α . It should be appreciated that the said 3-phosphoinositide-dependent protein kinase from one species or tissue can phosphorylate and activate PKB α from another species or tissue.

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By "activates protein kinase B α " we include the meaning that upon phosphorylation by the said 3-phosphoinositide-dependent protein kinase the activity of protein kinase B α to a given substrate increases by at least

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ten-fold compared to the protein kinase $B\alpha$ which has not been so phosphorylated, preferably by at least 20-fold and more preferably by at least 30-fold. Suitably, the activity of protein kinase $B\alpha$ is measured using the synthetic peptide RPRAATF.

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By "3-phosphoinositide-dependent protein kinase" we include the meaning that the protein kinase is substantially inactive at activating $PKB\alpha$ in the absence of a suitable 3-phosphoinositide (or a compound that mimics the effect of a 3-phosphoinositide). In particular, the said protein kinase has at least ten-fold increased activity towards protein kinase $B\alpha$ in the presence of a 3-phosphoinositide compared to the activity in the absence of said 3-phosphoinositide, preferably at least 100-fold, more preferably at least 1000-fold, and still more preferably at least 10,000-fold.

15 It will be appreciated that the 3-phosphoinositide-dependent protein kinase may be activated by mimics of 3-phosphoinositide as described in more detail below.

Preferably, the activation of $PKB\alpha$ by the 3-phosphoinositide-dependent protein kinase is substantially accelerated by the D-enantiomer of *sn*-1-stearoyl-2-arachidonyl phosphatidylinositol 3,4,5-trisphosphate but is not substantially accelerated by the L-enantiomer of the said phosphatidylinositol 3,4,5-trisphosphate.

25 Preferably, the 3-phosphoinositide-dependent protein kinase is substantially activated by the D-enantiomer of *sn*-1,2-dipalmitoyl phosphatidylinositol 3,4,5-trisphosphate or *sn*-1,2-dipalmitoyl phosphatidylinositol 3,4-bisphosphate but is not substantially activated by the L-enantiomers of the said phosphatidylinositol phosphates.

Preferably, the 3-phosphoinositide-dependent protein kinase is not substantially activated by phosphatidylinositol 3,5-bisphosphate or phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3-phosphate or inositol 1,3,4,5-tetrakisphosphate.

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Thus, particularly with reference to Figure 6 and the rabbit skeletal muscle PDK1 of Example 1, the following 3-phosphoinositides have been found to activate the said 3-phosphoinositide-dependent kinase (in order of level of activation; most effective first):

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1. Lipid 5: Racemic *sn*-1,2-dilinoleoyl PtdIns(3,4,5)P₃.
2. (Equal) Lipid 2: D-enantiomer of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P₃.
2. (Equal) Lipid 3: D-enantiomer of *sn*-2-arachidonyl-3-stearoyl
- 15 PtdIns(3,4,5)P₃.
4. (Equal) Lipid 6: *sn*-1,2 di-palmitoyl PtdIns(3,4,5)P₃.
4. Equal Lipid 7: *sn*-1,2 di-palmitoyl PtdIns(3,4)P₂.

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The following phospholipids cause no significant activation, at least in relation to rabbit skeletal muscle PDK1:

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6. Lipid 2: L-enantiomer of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P₃.
7. Lipid 4: L-enantiomer of *sn*-2-arachidonyl-3-stearoyl PtdIns(3,4,5)P₃.
8. Lipid 9: PtdIns(4,5)P₂.
9. Lipid 8: *sn*-1,2 di-palmitoyl PtdIns(3,5)P₂.
10. Lipid 11: *sn*-1,2 di-palmitoyl PtdIns-3P.
11. Lipid 10: PtdIns 4P.
12. IP₄: Ins(1,3,4,5)P₄.

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We have found that the human PDK1 enzyme has substantially the same

lipid preference as described above.

It is preferred if the 3-phosphoinositide-dependent protein kinase is substantially unaffected by wortmannin.

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It should be appreciated that the said 3-phosphoinositide-dependent protein kinase that phosphorylate and activates protein kinase B α is likely to phosphorylate and activate other forms of protein kinase B such as protein kinase B β and protein kinase B γ . We have shown that PDK1
10 phosphorylates and activates not only PKB α but also PKB β and PKB γ . A protein kinase B β is described in reference 7. A protein kinase B γ is described in Konishi *et al* (1995) *Biochem. Biophys. Res. Comm.* **216**, 526-534.

15 The 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α may be isolated from any convenient tissue and from any mammal as described below. It is believed that isoforms of the enzyme exist in different tissues within the same mammal and that the invention encompasses said isoforms and said 3-phosphoinositide-
20 dependent protein kinases from any mammal. It is preferred that the said 3-phosphoinositide-dependent protein kinase is the human said enzyme. It is also preferred if it is the rabbit said enzyme.

It is preferred if the said 3-phosphoinositide-dependent protein kinase is
25 about 67 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

A particularly preferred embodiment is a substantially pure 3-phosphoinositide-dependent protein kinase that phosphorylates and
30 activates protein kinase B α the 3-phosphoinositide-dependent protein

kinase comprising the amino acid sequences ANSFVGTAQYVSPELL or AGNEYLIFQK or LDHPFFVK or two or more of these sequences or amino sequences with from 1 to 4 conservative substitutions thereof. What is meant by "conservative substitutions" is described below.

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A particular preferred embodiment is a polypeptide which comprises the amino acid sequence

10 MARTTSQLYDAVPIQSSVVLCSPPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAEPRPGAGS
 LQHAQPPPQPRKKRPEDFKFGKILGEGSFSTVVLARELATSREYAIKILEKRHIKENKVPYVT
 RERDVMSRLDHPFFVKLYFTFQDDEKLYFGLSYAKNGELLKYIRKIGSFDETCTRFYTAETVSA
 LEYLHGKGIHRDLKPENILLNEDMHIQITDFGTAKVLSPEKQARANSFVGTAQYVSPELLTE
 KSACKSSDLWALGCIYQLVAGLPPFRAGNEYLIFQKIICKLEYDFPEKFFPKARDLVEKLLVLD
 ATKRLGCEEMEGYGPLKAHPFFESVTWENLHQQTTPPKLTAYLPAMSEDDDEDYGNYNLLSQFG
 15 CMQVSSSSSSSHLSASDTGLPQRSGSNIEQYIHDLSNSFELDLQFSEDEKRLLLLEKQAGGNPW
 HQFVENNLILKMGPVDKRKGLFARRRQLLLTEGPHLYYVDPVNKVLKGEIPWSQELRPEAKNFK
 TFFVHTPNRTYYLMDPSGNAHKWCRKIQEVWRQRYQSHPDAAVQ

or a variant, fragment, derivative or fusion thereof. The amino acid
 20 sequence is that of human PDK1 as determined from the nucleotide
 sequence of cDNAs encoding human PDK1.

The deduced amino acid sequence is also given in Figure 10.

25 As is discussed in more detail in the Examples, at least in relation to
 human PDK1, experiments in which the pleckstrin homology (PH)
 domains (which have been found to be present in human PDK1 and which
 are known to be present in PKB α and are believed to be involved in
 binding phospholipids) of either PDK1 or PKB α were deleted indicated
 30 that the binding of PtdIns(3,4,5)P3 or PtdIns(3,4)P2 to PKB α is required
 for phosphorylation and activation by PDK1. A GST-PKB α mutant
 lacking the PH domain possesses a 3-fold higher activity than that of full
 length wild-type GST-PKB α and was activated and phosphorylated by

PDK1 in a PtdIns(3,4,5)P3 independent manner; however, the rate of activation was reduced about 20-fold compared to wild-type GST-PKB α . A mutant PDK1 lacking the putative C-terminal PH domain, expressed as a GST-fusion protein, is still able to activate GST-PKB α in a PtdIns(3,4,5)P3-dependent manner, but the rate of activation was reduced about 30-fold compared to full length GST-PDK1. The effect of PtdIns(3,4,5)P3/PtdIns(3,4)P2 in the activation of PKB α by PDK1 is, therefore, at least partly substrate directed. However, the drastic reduction in the rate of activation of PKB by PDK1 when the PH domain of PDK1 is deleted indicates the importance of the PH domain of PDK1 and hence the importance of PtdIns(3,4,5)P3/PtdIns(3,4)P2 in the activation of PKB by PDK1. Thus, the substantially pure 3-phosphoinositide dependent protein lipase that phosphorylates and activates protein kinase B α may be 3-phosphoinositide dependent in the sense that the intact enzyme (for example intact PDK1) requires the presence of a 3-phosphoinositide in order to phosphorylate an intact PKB α *in vivo*. The protein kinase of the invention phosphorylates and activates PKB α in a 3-phosphoinositide-dependent manner.

PDK1 has been shown by us to phosphorylate p70 S6 kinase in the absence of InsPtd(3,4,5)P3. PDK1 has been shown to bind PtdIns(3,4,5)P3 directly.

A second aspect of the invention provides a recombinant polynucleotide encoding a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α provided that the recombinant polynucleotide is not the DNA corresponding to IMAGE clone 526583 or IMAGE clone 626511. The DNA sequence of at least part of the inserts of these clones are given in GenBank Accession No AA121994 and AA186323, respectively. Preferences for the said 3-

phosphoinositide-dependent protein kinase are the same as in the first aspect of the invention. The invention also encompasses polynucleotides which encode variants, fragments, derivatives or fusions of said 3-phosphoinositide-dependent protein kinase or fusions of the said variants, fragments or derivatives. The EST AA121994 was derived from pancreas tissue and the EST AA186323 was derived from HeLa cells.

A particular preferred embodiment of the invention is a polynucleotide comprising the nucleotide sequence

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10  ATGGCCAGGACCACCAGCCAGCTGTATGACGCCGTGCCCATCCAGTCCAGCGTGGTGTTA
    TGTTCCTGCCCATCCCATCAATGGTGAGGACCCAGACTGAGTCCAGCACGCCCCCTGGC
    ATTCCTGGTGGCAGCAGGCAGGGCCCCGCCATGGACGGCACTGCAGCCGAGCCTCGGCCC
    GGCGCCGGCTCCCTGCAGCATCCAGCCTCCGCCGCAGCCTCGGAAGAAGCGGCCTGAGG
    ACTTCAAGTTTGGGAAAATCCTTGGGGAAGGCTCTTTTCCACGGTTGTCTGGCTCGAG
15  AACTGGCAACCTCCAGAGAATATGCGATTAAATTCTGGAGAAGCGACATATCATAAAAG
    AGAACAAGGTCCCCTATGTAACCAGAGAGCGGGATGTATGTTCGCGCCTGGATCACCCCT
    TCTTTGTTAAGCTTTACTTCACATTTTCAGGACGACGAGAAGCTGTATTTTCGGCCTTAGTT
    ATGCCAAAATGGAGAATACTTAAATATATTTCGCAAAATCGGTTTCATTTCGATGAGACCT
    GTACCCGATTTTACACGGCTGAGATCGTGTCTGCTTTAGAGTACTTGCACGGCAAGGGCA
20  TCATTACAGGGACCTTAAACCGGAAAACATTTTGTTAAATGAAGATATGCACATCCAGA
    TCACAGATTTTGGAAACAGCAAAAGTCTTATCCCCAGAGAGCAAAACAAGCCAGGGCCAACT
    CATTTCGTGGGAACAGCGCAGTACGTTTCTCCAGAGCTGCTCACGGAGAAGTCCGCCTGTA
    AGAGTTCAGACCTTTGGGCTCTTGATGCATAATATACCAGCTTGTGGCAGGACTCCCAC
    CATTCCGAGCTGGAACGAGTATCTTATATTTTCTAGAAGATCATTAAAGTTGGAATATGACT
25  TTCCAGAAAATTCTTCCCTAAGGCAAGAGACCTCGTGGAGAACTTTTGGTTTTAGATG
    CCACAAAGCGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCTTAAAGCACACCCGT
    TCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCGAAGCTCACCGCTT
    ACCTGCCGGCTATGTTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGA
    GCCAGTTTGGCTGCATGCAGGTGTCTTCGTCCTCCTCACACTCCCTGTCAGCCTCCG
30  ACACGGGCCTGCCCCAGAGGTCAGGCAGCAACATAGAGCAGTACATTCACGATCTGGACT
    CGAACTCCTTTGAACTGACTTACAGTTTTCCGAAGATGAGAAGAGGTTGTTGTTGGAGA
    AGCAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAATACTAAAGATGG
    GCCCAGTGGATAAGCGGAAGGGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAG
    GACCACATTTATATTATGTGGATCCTGTCAACAAAGTTCTGAAAGGTGAAATTCCTTGGT
35  CACAAGAACTTCGACCAGAGGCCAAGAATTTTAAAACTTTCTTTGTCCACACGCCTAACA
    GGACGTATTATCTGATGGACCCCAGCGGGAACGCACACAAGTGGTGCAGGAAGATCCAGG
    AGGTTTGGAGGCAGCGATACCAGAGCCACCCGGACGCCGCTGTGCAGTGA

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or variants or variations thereof. The given nucleotide sequence is that

containing a coding sequence which encodes human PDK1.

The cDNA sequence is also given in Figure 10.

5 IMAGE clones 526583 and 626511 are known in the art and are publicly available from HGMP Resource Centre, I.M.A.G.E. Consortium, Hinxton, Cambridge CB1 1SB, UK. The clones are partial length cDNAs inserted into pBluescript SK-. It was not known that these clones were derived from a mRNA encoding a protein kinase.

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Certain other ESTs are publicly available which encode parts of the PDK1 cDNA namely H97903 (melanocyte), AA018098 (retina), AA18097 (retina), AA019394 (retina), AA019393 (retina), N22904 (melanocyte), W94736 (fetal heart), EST 51985 (gall bladder), N31292 (melanocyte),
15 AA188174 (HeLa cells), AA100210 (colon) and R84271 (retina). The polynucleotides corresponding to these ESTs are not claimed *per se* but they may be useful in carrying out certain parts of the invention. It had not been shown that these clones were derived from a mRNA encoding a protein kinase.

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The invention also includes a polynucleotide comprising a fragment of the recombinant polynucleotide of the second aspect of the invention. Preferably, the polynucleotide comprises a fragment which is at least 10 nucleotides in length, more preferably at least 14 nucleotides in length and
25 still more preferably at least 18 nucleotides in length. Such polynucleotides are useful as PCR primers.

The polynucleotide or recombinant polynucleotide may be DNA or RNA, preferably DNA. The polynucleotide may or may not contain introns in
30 the coding sequence; preferably the polynucleotide is a cDNA.

A "variation" of the polynucleotide includes one which is (i) usable to produce a protein or a fragment thereof which is in turn usable to prepare antibodies which specifically bind to the protein encoded by the said polynucleotide or (ii) an antisense sequence corresponding to the gene or to a variation of type (i) as just defined. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different amino acid that will not affect the activity or immunogenicity of the protein or which may improve or otherwise modulate its activity or immunogenicity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies and Applications of *In Vitro* Mutagenesis," *Science*, **229**: 193-210 (1985), which is incorporated herein by reference. Since such modified polynucleotides can be obtained by the application of known techniques to the teachings contained herein, such modified polynucleotides are within the scope of the claimed invention.

Moreover, it will be recognised by those skilled in the art that the polynucleotide sequence (or fragments thereof) of the invention can be used to obtain other polynucleotide sequences that hybridise with it under conditions of high stringency. Such polynucleotides includes any genomic DNA. Accordingly, the polynucleotide of the invention includes polynucleotide that shows at least 55 per cent, preferably 60 per cent, and more preferably at least 70 per cent and most preferably at least 90 per cent homology with the polynucleotide identified in the method of the invention, provided that such homologous polynucleotide encodes a polypeptide which is usable in at least some of the methods described below or is otherwise useful.

Per cent homology can be determined by, for example, the GAP program of the University of Wisconsin Genetic Computer Group.

DNA-DNA, DNA-RNA and RNA-RNA hybridisation may be performed
5 in aqueous solution containing between 0.1XSSC and 6XSSC and at temperatures of between 55°C and 70°C. It is well known in the art that the higher the temperature or the lower the SSC concentration the more stringent the hybridisation conditions. By "high stringency" we mean 2XSSC and 65°C. 1XSSC is 0.15M NaCl/0.015M sodium citrate.
10 Polynucleotides which hybridise at high stringency are included within the scope of the claimed invention.

"Variations" of the polynucleotide also include polynucleotide in which relatively short stretches (for example 20 to 50 nucleotides) have a high
15 degree of homology (at least 80% and preferably at least 90 or 95%) with equivalent stretches of the polynucleotide of the invention even though the overall homology between the two polynucleotides may be much less. This is because important active or binding sites may be shared even when the general architecture of the protein is different.

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By "variants" of the polypeptide we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the activity of the said 3-phosphoinositide-dependent protein kinase.

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By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

Such variants may be made using the methods of protein engineering and
30 site-directed mutagenesis well known in the art.

Preferably, the variant or variation of the polynucleotide encodes a 3-phosphoinositide-dependent protein kinase that has at least 30%, preferably at least 50% and more preferably at least 70% of the activity towards protein kinase B α of a natural said 3-phosphoinositide-dependent protein kinase, under the same assay conditions.

By "fragment of said 3-phosphoinositide-dependent protein kinase" we include any fragment which retains activity or which is useful in some other way, for example, for use in raising antibodies or in a binding assay.

By "fusion of said 3-phosphoinositide-dependent protein kinase" we include said protein kinase fused to any other polypeptide. For example, the said protein kinase may be fused to a polypeptide such as glutathione-S-transferase (GST) or protein A in order to facilitate purification of said protein kinase. Fusions to any variant, fragment or derivative of said protein kinase are also included in the scope of the invention.

A further aspect of the invention provides a replicable vector comprising a recombinant polynucleotide encoding a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α , or a variant, fragment, derivative or fusion of said protein kinase or a fusion of said variant, fragment or derivative.

A variety of methods have been developed to operably link polynucleotides, especially DNA, to vectors for example *via* complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to

form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA
5 segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

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The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as
15 bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

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Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

25 A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. This method may be used for introducing the DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful
30 ways as is known in the art.

In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors
5 using methods known in the art.

The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention. Thus, the DNA encoding the polypeptide constituting the
10 compound of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in
15 US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July
20 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA (or in the case of retroviral vectors, RNA) encoding the
25 polypeptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial

host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur.

5 Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and
10 pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTrc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late
15 promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible
20 promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037,
25 USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

30 The present invention also relates to a host cell transformed with a

polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic and kidney cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650 and 293 cells which are human embryonic kidney cells. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life

Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cell, bacterial cells,
5 insect cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants
10 is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25 μ FD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* 194, 182.

15 Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the
20 invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

25 In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an
30 expression vector produce proteins displaying appropriate antigenicity.

Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

A further aspect of the invention provides a method of making a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase $B\alpha$ or a variant, derivative, fragment or fusion thereof or a fusion of a variant, fragment or derivative the method comprising culturing a host cell comprising a recombinant polynucleotide or a replicable vector which encodes said 3-phosphoinositide-dependent protein kinase, and isolating said protein kinase or a variant, derivative, fragment or fusion thereof of a fusion of a variant, fragment or derivative from said host cell. Methods of cultivating host cells and isolating recombinant proteins are well known in the art.

A still further aspect of the invention provides a method of isolating a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase $B\alpha$ the method comprising the steps of (a) obtaining tissue from a mammal that contains said 3-phosphoinositide-dependent kinase, (b) obtaining a cell-free extract from said tissue, (c) fractionating said cell-free extract and (d) selecting a fraction from step (c) which is capable of phosphorylating and activating protein kinase $B\alpha$ in the presence of a 3-phosphoinositide.

Preferably, the 3-phosphoinositide is any of the preferred 3-phosphoinositides as disclosed in relation to the first aspect of the

invention; most preferably it is phosphatidylinositol-3,4,5-trisphosphate or phosphatidylinositol-3,4-bisphosphate. Preferably, further steps are employed. Conveniently, in a step (e) the fraction of step (d) is fractionated further and in a step (f) a fraction is selected from step (e) which is capable of phosphorylating and activating protein kinase B α in the presence of a 3-phosphoinositide. Steps (e) and (f) may be repeated until a substantially pure preparation of a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α is obtained. Suitably, the fractionation steps include any of ion exchange chromatography, polyethylene glycol (PEG) precipitation, heparin chromatography or any other fractionation procedures. Preferably, the method steps are those substantially as described in Example 1.

Conveniently, the tissue is skeletal muscle. The tissue may be from any mammal including humans.

A further aspect of the invention provides a phosphoinositide-dependent protein kinase which can phosphorylate and activate protein kinase B α , or a variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative obtainable by the methods herein disclosed.

A still further aspect of the invention provides an antibody reactive towards a phosphoinositide-dependent protein kinase of the invention.

Antibodies reactive towards the said phosphoinositide-dependent protein kinase of the invention may be made by methods well known in the art. In particular, the antibodies may be polyclonal or monoclonal.

Suitable monoclonal antibodies which are reactive towards the said protein kinase may be prepared by known techniques, for example those disclosed

in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", SGR Hurrell (CRC Press, 1982).

- 5 In a preferred embodiment the antibody is raised using any one of the peptide sequences ANSFVGTAQYVSPPELL or AGNEYLIFQK or LDHPFFVK. It is preferred if polyclonal anti-peptide antibodies are made. Other peptides may be used to make antibodies, for example the peptides RQRYQSHPDAAVQ and LSPESKQARANS.

10

Peptides in which one or more of the amino acid residues are chemically modified, before or after the peptide is synthesised, may be used providing that the function of the peptide, namely the production of specific antibodies *in vivo*, remains substantially unchanged. Such modifications

15 include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from *in vivo* metabolism. The peptides may be present as single copies

20 or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently antigenic themselves to obviate the use of a carrier. It may be advantageous for the peptide to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow

25 disulphide bonds to be formed. If the peptide is covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the peptide of the invention forms a loop.

According to current immunological theories, a carrier function should be

30 present in any immunogenic formulation in order to stimulate, or enhance

- stimulation of, the immune system. It is thought that the best carriers embody (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys, beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or different peptides of the invention may be cross-linked to one another; in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal cysteine residue (if present).
- If the peptide is prepared by expression of a suitable nucleotide sequence in a suitable host, then it may be advantageous to express the peptide as a fusion product with a peptide sequence which acts as a carrier. Kabigen's "Ecosec" system is an example of such an arrangement.
- The peptide of the invention may be linked to other antigens to provide a dual effect.

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded

by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally

available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

10 A further aspect of the invention provides a method of identifying a compound that modulates the activity of a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase $B\alpha$, the method comprising contacting a compound with the said 3-phosphoinositide-dependent protein kinase or a variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof and determining whether, in the presence of said compound, phosphorylation and activation of a protein kinase B or phosphorylation a suitable substrate of the 3-phosphoinositide-dependent protein kinase is changed compared to the activity of said 3-phosphoinositide-dependent protein kinase or said variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof in the absence of said compound.

We believe that the said 3-phosphoinositide-dependent protein kinase of the invention is able to phosphorylate not only protein kinase $B\alpha$ but also other forms of protein kinase B such as protein kinase $B\beta$ and protein kinase $B\gamma$. We have shown that PDK1 is able to phosphorylate $PKB\alpha$, $PKB\beta$ and $PKB\gamma$. Thus, in this method and the methods described below the protein kinase B can be any protein kinase B such as protein kinase $B\alpha$ or protein kinase $B\beta$ or protein kinase $B\gamma$. Other substrates of the 3-

phosphoinositide-dependent protein kinase may be used in the assay method of the invention, for example p70 S6 kinase. A suitable phospholipid such as PtdIns(3,4,5)P3 is typically present in the screen when a PKB containing a PH domain is used as a substrate but it is not
5 necessary for a 3-phosphoinositide to be present in some circumstances, for example when p70 S6 kinase, or PKB lacking a PH domain, is used as a substrate. Phosphorylation of p70 S6 kinase occurs in the absence of PtdIns(3,4,5)P3.

10 It will be appreciated that the method can be carried out *in vitro* using 3-phosphoinositide-dependent protein kinase or a variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative in the presence of a suitable protein kinase B or other suitable substrate. But it will also be appreciated that it may be carried out *in vivo*, for
15 example using the yeast two-hybrid system to detect compounds which reduce or enhance the interactions between the phosphoinositide-dependent protein kinase and PKB or another suitable substrate.

In one embodiment the compound decreases the activity of the 3-
20 phosphoinositide-dependent protein kinase.

In another embodiment the compound increases the activity of the 3-phosphoinositide-dependent protein kinase. Preferably the compound competes with 3-phosphoinositide. Preferably, the compound substantially
25 reduces activation by phosphatidylinositol-3,4,5-trisphosphate or phosphatidyl-inositol-3,4-bisphosphate. Preferably, the compound substantially enhances activation by phosphatidylinositol-3,4,5-trisphosphate or phosphatidylinositol-3,4-bisphosphate. It will be appreciated that the method may be used to identify compounds which
30 bind to and effect the activity of either the said 3-phosphoinositide-

dependent protein kinase or the protein kinase B when protein kinase B is present in the assay.

A still further aspect of the invention provides a method of identifying a compound that mimics the effect of a 3-phosphoinositide on a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α , the method comprising determining whether said compound activates a said 3-phosphoinositide-dependent protein kinase or a suitable variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative so that it can phosphorylate and activate protein kinase B α or phosphorylate any other suitable substrate, the activation by said compound being in the absence of a 3-phosphoinositide.

The activity of protein kinase B can be measured using any suitable substrate; for example, the peptide RPRAATF is a preferred substrate but, for example, myelin basic protein and certain histones are also substrates of PKB.

Preferably, the 3-phosphoinositide is phosphatidylinositol-3,4,5-trisphosphate or phosphatidylinositol-3,4-bisphosphate.

We believe that PtdIns(3,4,5)P3 or a suitable phosphoinositide interacts with the PH domain of a PKB in order for PDK1 to phosphorylate Thr-308 of PKB α and activate this protein kinase. Our data suggests that a PKB molecule lacking the PH domain is activated and phosphorylated independently of PtdIns(3,4,5)P3. There are several mechanisms by which a drug (which interferes with the interaction between the kinase of the invention, for example, PDK1 and PKB) may act. In one mechanism it may bind to PKB and prevent PKB from becoming activated by the

kinase of the invention, for example PDK1. In the other mechanism, the drug may bind to the kinase of the invention (such as PDK1) and inhibit PDK1 activity. In a further mechanism the drug may bind to PDK1 and prevent it activating PKB. It is preferred if the screening assays of the invention are carried out using both a full-length PKB molecule (for example a GST-PKB fusion) or a molecule which, although not full length, retains a PH domain which is substantially capable of binding a suitable phospholipid such as PtdIns(3,4,5)P3, and a PKB in which the PH domain has been modified so that the PKB is substantially incapable of binding a suitable phospholipid such as PtdIns(3,4,5)P3. Suitable modifications include deletion of all or part of the PH domain or mutations in the PH domain which substantially prevent binding of the suitable phospholipid. Compounds which prevent PtdIns(3,4,5)P3 from interacting with PKB are particularly selected, which can be detected in the screen.

Alternatively, it may be useful to use PKB lacking a functional PH domain in a screening assay of the invention, in this case no 3-phosphoinositide need be used in order for PDK1 to activate PKB.

A further aspect of the invention provides a compound identifiable by the screening methods disclosed herein.

The methods effectively are screening assays for compounds which modulate the said 3-phosphoinositide-dependent protein kinase or its interactions with a 3-phosphoinositide or with a protein kinase B.

Thus, the screening methods of the invention include methods for identifying compounds which compete with the 3-phosphoinositide (in particular phosphatidylinositol-3,4,5-trisphosphate or phosphatidylinositol-3,4-bisphosphate) and which lead to inactivation or activation of the said

3-phosphoinositide-dependent protein kinase or other modulation of the enzyme activity; they also include screening methods for substances which bind to the said 3-phosphoinositide-dependent protein kinase and which reduce or enhance the activation of a protein kinase B or another suitable substrate (for example, they may bind to said 3-phosphoinositide-dependent protein kinase and prevent it interacting with a protein kinase B); and they also include screening methods for substances which bind to a protein kinase B and which reduce or enhance its interaction with said 3-phosphoinositide-dependent protein kinase. The screening methods of the invention also include methods for identifying compounds which block the catalytic sites of PKB and the protein kinase of the invention (for example, PDK1). Methods for carrying out this type of screening assay are well known in the art.

It will be appreciated that in those assays where a said 3-phosphoinositide-dependent protein kinase is required and the phosphorylation of a particular substrate requires the presence of a 3-phosphoinositide, a 3-phosphoinositide is present. Any suitable 3-phosphoinositide is useful but it is preferred if the 3-phosphoinositide is phosphatidylinositol-3,4,5-trisphosphate or phosphatidylinositol-3,4-bisphosphate. However, as is clear from the foregoing, some assay systems do not require the presence of a 3-phosphoinositide such as PtdIns(3,4,5)P₃.

The compounds identified in the methods may themselves be useful as drug or they may represent lead compounds for the design and synthesis of more efficacious compounds which modulate the activity of the said 3-phosphoinositide-dependent protein kinase or its interactions with a 3-phosphoinositide or with a protein kinase B.

Thus, a further aspect of the invention provides a method of modulating

in a cell the activity of the said 3-phosphoinositide-dependent protein kinase or its interactions with a 3-phosphoinositide or with a protein kinase B, the method comprising introducing into the cell a compound identifiable in the screening assay described above. Preferably, the cell is in a human patient.

Compounds, identifiable in the screening method, which mimic the effect of a 3-phosphoinositide, preferably phosphoinositol-3,4,5-trisphosphate or phosphoinositol-3,4-bisphosphate, are believed to be useful in treating diabetes. Compounds identifiable in the screening methods of the invention that inhibit PKB, PDK1 or the activation of PKB by PDK1 are believed to be useful in treating cancer. PKB is the cellular homologue of v-akt which is involved in leukaemias. Two isoforms of PKB are overexpressed in ovarian, pancreatic and breast cancers. It is believed that PKB mediates protection of cells to apoptosis mediated, for example, by IGF-1. Overexpression of PKB may allow cancer cells to proliferate by stopping apoptosis.

It will be appreciated that certain compounds found in the screening methods may be able to enhance cell proliferation in a beneficial way and may be useful, for example in the regeneration of nerves or in wound healing.

Further aspects of the invention provide the use of said 3-phosphoinositide-dependent protein kinase or variants, fragments, derivatives or fusions thereof or fusions of said variants, fragments or derivatives in a screening assay for compounds which modulate the activity of said protein kinase, in particular its interaction with 3-phosphoinositide or a protein kinase B. The invention also includes the use of the said 3-phosphoinositide-dependent protein kinase, such as

PDK1, to phosphorylate and active protein kinase B and it includes a method of activating PKB using a 3-phosphoinositide-dependent protein, such as PDK1.

5 A still further aspect of the invention provides kits of parts that are useful in carrying out the screening methods. Conveniently, the kit of parts comprises a said 3-phosphoinositide-dependent protein kinase or a suitable variant or fragment or derivative or fusion thereof or a fusion of a variant or fragment or derivative (or a polynucleotide which encodes any of these)
10 and a protein kinase B or a suitable variant or fragment or derivative or fusion thereof or a fusion of a variant or fragment or derivative (or a polynucleotide which encodes any of these). It will be appreciated that, depending on the screening method employed, it may additionally comprise a suitable 3-phosphoinositide or a suitable substrate for the
15 protein kinase B.

Abbreviations: PKB, Protein kinase B; PtdIns(3,4,5)P3, Phosphatidylinositol 3,4,5-tris phosphate; Ptdins(3,4,)P2, Phosphatidylinositol 3,4-bisphosphate; PI 3-kinase, Phosphoinositide 3-
20 kinase; PtdCho, Phosphatidylcholine; PtdSer, Phosphatidylserine; PH, pleckstrin homology.

The invention will now be described in more detail with reference to the following Examples and Figures wherein

25

Figure 1. SDS Polyacrylamide gel of purified GST-PKB α . 293 cells were transiently transfected with the pEBG2T DNA construct expressing GST-PKB α , serum starved for 16 h and, after cell lysis, GST-PKB α was purified by affinity chromatography on glutathione-Sepharose (see
30 Methods). "Sepharose" is a trade mark. The glutathione-Sepharose

eluate (3 μ g protein) was electrophoresed on a 10% SDS polyacrylamide gel and stained with Coomassie blue. "Coomassie" is a trade mark. The position of the molecular mass markers, glycogen phosphorylase (97 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) are indicated.

5

Figure 2. Identification and purification of a PtdIns(3,4,5)P₃ dependent protein kinase (PDK1) which activates GST-PKB α . Following the PEG precipitation PDK1 was chromatographed sequentially on SP-Sepharose (A) and heparin-Sepharose (B) and Peak 2 from the latter
10 column was chromatographed on Mono S (C) (see Methods). "Mono S" is a trade mark. PDK1 activity was assayed either in the presence of phospholipid vesicles comprising 100 μ M PtdCho, 100 μ M PtdSer, 10 μ M D-enantiomer of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P₃ (open circles) or vesicles comprising only 100 μ M PtdCho and 100 μ M PtdSer (closed
15 circles). The broken lines indicate the salt gradient and the solid line the absorbance at 280 nm. The amount of protein eluted from the Mono S column was too low see any absorbance at 280 nm.

Figure 3. PDK1 phosphorylates and activates GST-PKB α . (A) GST-PKB α (0.5 μ M) was incubated with PDK1 (12 U/ml), 10 mM Mg(Ac)₂,
20 100 μ M [γ ³²P]ATP, and vesicles comprising 100 μ M PtdCho, 100 μ M PtdSer, 10 μ M D-enantiomer of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P₃ in Buffer B. At various times, aliquots were removed and either assayed for PKB α activity (closed circles) or for incorporation of
25 phosphate into PKB α (open circles). Activities are presented relative to control experiments in which PDK1 was omitted from the reaction mixture. Phosphorylation was assessed by counting the ³²P-radioactivity associated with the band of GST-PKB α after SDS-PAGE. (B) Same as (A) except that the effects of omitting PDK1, ATP, PtdSer/PtdCho,
30 Ptdins(3,4,5)P₃, heating PDK1 at 55°C for 2 min, or adding 0.5% (by

vol) Triton X-100 in the PDK1 assay were studied. The assays were carried out for 60 min. Closed bars show GST-PKB α activity and hatched bars GST-PKB α phosphorylation relative to a control assay in which PDK1, ATP, PtdCho/PtdSer and PtdIns(3,4,5)P3 were present. The results are presented as \pm SEM for six determinations (two independent experiments).

Figure 4. Dependence of PDK1 activity on PtdIns(3,4,5)P3 concentration. GST-PKB α (0.5 μ M) was incubated with PDK1 (12 U/ml), 10 mM MgAc, 100 μ M [γ^{32} P]ATP and the indicated concentrations of D-enantiomer of 1-stearoyl-2-arachidonyl Ptdins(3,4,5)P3 in either the presence of constant concentration of PtdCho and PtdSer (100 μ M of each open circles) or in the presence of a 10 fold molar excess of the PtdSer/PtdCho (closed circles). After 30 min the extent of activation (A) and phosphorylation (B) of GST-PKB α was assessed as described in Materials and Methods. The results are presented as \pm SEM for 2 experiments, each carried out in triplicate.

Figure 5. PDK1 phosphorylates PKB α at Thr-308. (A) GST-PKB α was phosphorylated by incubation for 60 min with PDK1 (12 U/ml), and phospholipid vesicles comprising PtdCho, PtdSer, D-enantiomer of 1-stearoyl-2-arachidonyl Ptdins(3,4,5)P3, and Mg[γ^{32} P]ATP (see legend to Fig 3), and then alkylated and digested with trypsin (see Methods). The digest was applied to a Vydac 218TP54 C18 column (Separations Group, Hesperia, CA) equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA) in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and fractions of 0.4 ml were collected. 35% of the radioactivity applied to the column was recovered from the major 32 P-containing peptide at 26% acetonitrile (the remainder of the radioactivity eluted as numerous minor peaks). The elution

positions of the tryptic peptides which contain Thr-308 and Ser-473 are marked [15]. (B) A portion of the major ^{32}P -containing peptide (500 cpm) from A was coupled covalently to a Sequelon arylamine membrane and analysed on an Applied Biosystems 470A sequencer using the modified
 5 programme described in [30]. ^{32}P radioactivity was measured after each cycle of Edman degradation.

Figure 6. PDK-1 is activated specifically and stereospecifically by PtdIns(3,4,5)P3 and PtdIns(3,4)P2 lipids. GST-PKB α was incubated for
 10 30 min at 30°C with Mg[γ ^{32}P]ATP in the presence (filled bars) and absence (open bars) of PDK1 and phospholipid vesicles containing 100 μM PtdCho, 100 μM PtdSer, and various PtdIns lipids (numbered 1-11, see below) or Ins (1,3,4,5)P4, all at a final concentration of 10 μM in the assay. Reactions were terminated by the addition of 1% (by vol) Triton
 15 X-100 to the assay and in (A) the activity of GST-PKB α was determined as described under Methods. (B) The phosphorylation of GST-PKB α was assessed by autoradiography of the Commassie blue-stained band corresponding to GST-PKB α (Fig 1). The results are presented as fold-activation of GST-PKB α \pm SEM for 6-8 determinations (three
 20 independent experiments). Lipids 1 and 2 are the D and L-enantiomers of *sn*-1-stearoyl-2-arachidonyl Ptdins(3,4,5)P3 respectively, and lipids 3 and 4 are the D and L-enantiomers of *sn*-2-arachidonyl-3-stearoyl Ptdins(3,4,5)P3 respectively. Lipid 5 is racemic *sn*-1,2-dilinoleoyl Ptdins(3,4,5)P3. The remaining lipids are all D-enantiomers. Lipid 6 is
 25 *sn*-1,2 di-palmitoyl Ptdins(3,4,5)P3, lipid 7 is *sn*-1,2 di-palmitoyl Ptdins(3,4)P2, lipid 8 is *sn*-1,2 di-palmitoyl Ptdins(3,5)P2. Lipid 9 is Ptdins(4,5)P2, and lipid 10 is PtdIns 4P which are both purified lipid derived from Folch fraction type-I brain extract. Lipid 11 is *sn*-1,2 di-palmitoyl PtdIns-3P. IP4 is Ins(1,3,4,5)P4.

Figure 7. Proposed mechanism by which insulin induces the activation of PKB α . The activation of the insulin receptor by insulin causes the receptor to phosphorylate itself at several tyrosine residues. This leads to the docking of insulin receptor substrate-1 (IRS-1) and IRS-2 and their phosphorylation at multiple tyrosine residues by the insulin receptor. Several phosphorylated tyrosine residues on IRS1 and IRS2 then interact with SH2 domains on the p85 subunit of PI 3-kinase, leading to the recruitment of the p110 catalytic subunit of PI 3-kinase to cell membranes and its activation [31]. PI 3-kinase then phosphorylates Ptdins(4,5)P2 at the D-3 position of the inositol ring, resulting in the formation of the second messenger Ptdins(3,4,5)P3 whose levels (in a typical cell) rise from 0.1% to 1-2% of the PtdIns(4,5)P2 content in the plasma membrane [31]. PtdIns(3,4,5)P3 interacts with, and activates PDK1 which partially activates PKB α by phosphorylating it at Thr-308. Maximal activation of PKB α also requires the phosphorylation of Ser-473 by an unknown kinase. The insulin-induced phosphorylation of Ser-473, like the phosphorylation of Thr-308, is prevented by inhibitors of PtdIns 3-kinase [15], suggesting that Ser-473 may be phosphorylated by a distinct 3-phosphoinositide-dependent protein kinase (PDK2). However, the Ser-473 kinase may be activated indirectly by 3-phosphoinositides by mechanisms discussed in the text. For this reason, the pathway from PtdIns(3,4,5)P3 to the phosphorylation of Ser-473 is shown by broken lines with a question mark after PDK2. In skeletal muscle, the activation of PKB α may increase the rate of glucose uptake [9] and glycogen synthesis [7] and stimulate protein translation [8]. In neurons and other cells PKB α activation may provide a survival signal by suppressing apoptosis [10,11].

Figure 8. Details of IMAGE Consortium clone ID 526583.

Figure 9. Details of IMAGE Consortium clone ID 626511.

Figure 10. Nucleotide sequence coding for, and deduced amino acid sequence of, PDK1.

Figure 11. Alignment of the amino acid sequences of human PDK1 and *Drosophila* DSTPK61. The alignment was carried out using the Clustal W program [46]. Asterisks indicate identities between PDK1 and STK61. The catalytic domain comprises residues 83-342 of PDK1 and residues 165-487 of STPK61. The putative PH domains lie between residues 450 and 550 of PDK1 and 581 and 684 of STPK61B.

Figure 12. GST-PDK1 and GST-DSTPK61 activate and phosphorylate PKB α in a PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ dependent manner. GST-PKB α was incubated for 30 min at 30°C with either 5 U/ml GST-PDK1 (panels A, B) or GST-DSTPK61 (panels C, D) with Mg[γ ³²P]ATP and phospholipid vesicles containing 100 μ M PtdCho, 100 μ M PtdSer, and various PtdIns lipids (numbered 1-5, see below) all at a final concentration of 10 μ M in the assay. Under these conditions, the increase in PKB α activity and phosphorylation was linear with respect to time (see Methods). In panels A and C, the assays were terminated by making the incubations 1% (by vol) in Triton X-100 (see Ref 21), and the increase in specific activity of GST-PKB α was determined. In panels B and D, the reactions were terminated by making the solutions 1% in SDS, the samples were subjected to SDS/polyacrylamide gel electrophoresis, and the phosphorylation was assessed by autoradiography of the Coomassie blue-stained band corresponding to GST-PKB α . The results are presented in panels A and C as the increase in the specific activity of GST-PKB α (U/mg), relative to a control incubation in which GST-PDK1 or GST-DSTPK61 was omitted (\pm SEM for 6-9 determinations, three independent experiments). Under all conditions the increase in both activity and phosphorylation of GST-PKB α was linear with time. Track 1, buffer

control; Track 2, *sn*-1-stearoyl-2-arachidonyl-D-Ptdins(3,4,5)P₃; Track 3, *sn*-1,2 di-palmitoyl-D-Ptdins(3,4)P₂; Track 4 is PtdIns(4,5)P₂ (purified from Folch brain fraction); Track 5, *sn*-1,2 di-palmitoyl-D-PtdIns-3P.

5 **Figure 13. PDK1 phosphorylates PKB α at Thr308.** GST-PKB α was maximally phosphorylated by incubation for 30 min with 25 U/ml of either GST-PDK1 (A) or GST-DSTPK61 (B) and phospholipid vesicles comprising PtdCho, PtdSer, the D-enantiomer of 1-stearoyl-2-arachidonyl PtdIns(3,4,5)P₃, and Mg[γ ³²P]ATP (10⁶ cpm per nmol). After 30 min, the
10 samples were alkylated with 4-vinylpyridine, digested with trypsin [21] and applied to a Vydac 218TP54 C₁₈ column (Separations Group, Hesperia, CA) equilibrated in 0.1 % (v/v) trifluoroacetic acid (TFA). The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and fractions of 0.4 ml were collected. 38 %
15 (panel A) and 45 % (panel B) of the radioactivity applied to the column was recovered in the major ³²P-containing peptide eluting at 26 % acetonitrile, while contains Thr308. The elution position of the typic peptide containing Ser473 is also marked [see ref 20 and Fig 15].

20 **Figure 14. PKB α is activated by cotransfection with PDK1 in 293 cells.** (A) 293 cells were transiently transfected with DNA constructs expressing either HA-PKB α or both HA-PKB α and Myc-PDK1. The cells were then stimulated for 10 min with or without 50 ng/ml IGF1, the HA-PKB α immunoprecipitated from the lysates and assayed. The results are
25 expressed relative to the specific activity of wild type HA-PKB α in unstimulated 293 cells (0.03 \pm 0.5 U/mg). No PKB α activity was detected in mock transfections in which the cells were transfected with PCMV5 vector alone (data not shown). (B) 6 μ g of protein from each lysate was electrophoresed on a 10 % SDS/polyacrylamide gel and
30 immunoblotted using either a monoclonal HA-antibody to detect HA-

PKB α or a monoclonal Myc-antibody to detect Myc-PDK1. The molecular mass markers are glycogen phosphorylase (97 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa).

5 **Figure 15. Overexpression of PDK1 in cells induces phosphorylation of PKB α at Thr308.** 293 cells transiently transfected with either wild type HA-PKB α (panels A and C) or HA-PKB α and Myc-PDK1 (panels B and D) were incubated with 32 P-orthophosphate, then treated for 10 min without (panels A and B) or with (panels C and D) 50 ng/ml IGF1. The
10 32 P-labelled HA-PKB α was immunoprecipitated from the lysates, treated with 4-vinylpyridine, and electrophoresed on a 10% polyacrylamide gel. The HA-PKB α was excised from the gel, digested with trypsin and chromatographed on a C₁₈ column (see legend to Fig 13) to resolve the four major phosphopeptides which are phosphorylated at Ser-124, Ser473,
15 Thr308 and Thr450 respectively [20]. The identity of each phosphopeptide was established by phospho-amino acid analysis and solid phase sequencing. Similar results were obtained in two separate experiments for (panels A and C), and in three separate experiments (panels B and D).

20

Figure 15. Role of the PH domains of PKB α and PDK1. (A) 0.5 μ g of either GST-PKB α or GST- Δ PH-PKB α were incubated for 30 min at 30°C with either 2.3 nM GST-PDK1 (residues 1-556) with Mg[γ - 32 P]ATP and phospholipid vesicles containing 100 μ M PtdCho, 100 μ M PtdSer
25 either in the presence or absence of 10 μ M PtdIns(3,4,5)P3 in a 20 μ l assay. The assays were terminated by making the incubations 1% (by vol) in Triton X-100 as in Example 1, and the increase in specific activity of GST-PKB α was determined. The basal activity of GST-PKB α was 2.5 U/mg and that of GST- Δ PH-PKB α was 8 U/mg. At the high
30 concentration of GST-PDK1 used in this assay the rate of activation of full

length wild type GST-PKB α is not linear with time whereas the activation of GST- Δ PH-PKB α is linear. When the experiments are carried out at 20-fold lower concentrations of GST-PDK1 a similar extent of activation of wild-type GST-PKB α is achieved as that shown in the figure for GST- Δ PH-PKB α by 2.3 nM GST-PDK1. (B) as in (A) except that 0.5 μ g of GST-PKB α was incubated with 2.5 nM GST- Δ PH-PDK1 (residues 1 to 450) in the presence of phospholipid vesicles containing 100 μ M PtdCho, 100 μ M PtdSer and 10 μ M of either, *sn*-1-stearoyl-2-arachidonyl-D-PtdIns(3,4,5)P3 (Track 1); *sn*-1-stearoyl-2-arachidonyl-L-PtdIns(3,4,5)P3 (Track 2), *sn*-1,2 di-palmitoyl-D-PtdIns(3,4,5)P3 (Track 3), *sn*-1,2 di-palmitoyl-D-PtdIns(3,4,5)P3 (Track 4), PtdIns(4,5)P2 (purified from Folch brain fraction Track 5) and *sn*-1,2 di-palmitoyl-D-PtdIns-3P (Track 6). The results are presented as the increase in the specific activity of GST-PKB α (U/mg), (\pm SEM for 6-9 determinations, three independent experiments), relative to a control incubation in which GST-PDK1 is omitted.

Figure 17. Evidence that PDK1 and DSTPK61 possess a Pleckstrin-Homology domain. Sequence alignment of PH domains that have a known tertiary structure with the putative PH domains of PDK1 and DSTPK61. Alignment was done using the program AMPS (Barton & Sternberg (1990) *J. Mol. Biol.* 212, 389-402) and formatted using AMAS (Livingstone & Barton (1993) *Comp. Appl. Bio. Sci.* 9, 745-756) with some manual adjustment to ensure coincidence of secondary structure regions. Regions containing conserved residues are coloured green and the invariant tryptophan (Trp-535) is coloured red. General regions of secondary structure are denoted by the blue (beta strand) and green (alpha helix) bands. The Pleckstrin-Homology sequences are those found in human Pleckstrin, PLS; human spectrin, SPC; human dynamin DYN, and rat phospholipase C- δ , PLC δ . Numbering is based on the PDK1 sequence

(Fig 10).

Figure 18. SDS Polyacrylamide gel of purified GST-PDK1 and GST-DSTPK61. GST-PDK1 encoding residues 52-556, Lane 1. 3 μ g protein) and GST-DSTPK61 (Lane 2. 6 μ g protein) were electrophoresed on 1 7.5% SDS polyacrylamide gel and stained with Coomassie blue. The position of the molecular mass markers, glycogen phosphorylase (97 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) are indicated. The other GST-PDK1 constructs used in this study were all expressed at 10 a similar level of purity as the GST-PDK1 preparation shown in Lane 1 (data not shown).

Example 1: Purification and characterisation of a 3-phosphoinositide-dependent protein kinase (PDK1) that phosphorylates and activates protein kinase B α 15

Summary

Background: Protein kinase B (PKB), also known as c-Akt, is activated 20 rapidly when cells are stimulated with insulin and growth factors, and much of the current interest in this enzyme stems from the observation that it lies downstream of phosphoinositide 3-kinase. We recently showed that insulin or IGF1 induce the phosphorylation of PKB at Thr-308 and Ser-473. The phosphorylation of both residues is required to activate PKB 25 maximally, and the insulin or IGF1-induced phosphorylation of both residues is prevented by incubating cells with inhibitors of PI-3 kinase.

Results: A protein kinase has been purified 500,000-fold from rabbit skeletal muscle extracts which phosphorylates protein kinase B α (PKB α) 30 at Thr-308 and increases its activity >30-fold. The kinase was only

active in the presence of low micromolar concentrations of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] or phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P2] and has therefore been termed PtdIns(3,4,5)P3-dependent protein kinase-1 (PDK1). The D-enantiomers of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P3 and *sn*-2-arachidonyl-3-stearoyl Ptdins(3,4, 5)P3 potently activated PDK1, but the L-enantiomers of these derivatives were almost ineffective. *sn*-1,2-dipalmitoyl PtdIns(3,4,5)P3 and *sn*-1,2-dipalmitoyl PtdIns(3,4,)P2 both activated PDK1 to a similar extent, but were ~3-fold less effective than the *sn*-1-stearoyl-2-arachidonyl and *sn*-2-arachidonyl-3-stearoyl derivatives. PtdIns(3,5)P2, PtdIns(4,5)P2, PtdIns(4)P, PtdIns(3)P and inositol 1,3,4,5-tetrakisphosphate did not activate PDK1 at all. None of the inositol phospholipids tested activated or inhibited PKB α or induced its phosphorylation under the same conditions. PDK1 activity was unaffected by wortmannin, indicating that it is not likely to be a PI 3-kinase family member.

Conclusions: PDK1 is likely to be one of the protein kinases which mediate the activation of PKB by insulin and growth factors. PDK1 may therefore play a key role in mediating many of the actions of the second messengers, PtdIns(3,4,5)P3 and/or PtdIns(3,4)P2.

Materials and Methods

Materials. PtdSer (pig brain) was purchased from Doosan Serdary Research Laboratories (New Jersey, USA) and *sn*-1-stearoyl-2-arachidonyl PtdCho from Sigma (Poole, UK). PtdIns 4P and PtdIns(4,5)P2 were purified as described previously from Folch fraction type-1 extract of bovine brain (Sigma) [26]. Synthetic *sn*-1,2-dipalmitoyl analogues of PtdIns(3,4,5)P3, PtdIns(3,4)P2, PtdIns(3,5)P2 and PtdIns 3P were made

as described previously[18]. Synthetic D and L enantiomers of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P₃, *sn*-2-arachidonyl-3-stearoyl PtdIns(3,4,5)P₃ and the D enantiomer of *sn*-1-linoleoyl-2-linoleoyl PtdIns(3,4,5)P₃ were synthesised from inositol (Gaffney and Reece 1997, manuscript submitted for publication). All phospholipids were 97-98% pure. Synthetic phosphatidylinositol bisphosphates were stored at -20°C as solutions in dimethyl sulphoxide (DMSO) and phosphatidylinositol trisphosphates in either DMSO or water. All other phospholipids were stored in chloroform-methanol solvents. The peptide used to assay PKB α , (RPRAATF) [16] and TTYADFLASGRTGRRNAIHD (the specific peptide inhibitor of cyclic AMP dependent protein kinase, termed PKI) were synthesised by Mr F. Barry Caudwell (MRC Protein Phosphorylation Unit, Dundee) on an Applied Biosystems 431A peptide synthesiser, and their concentrations were determined by quantitative amino acid analysis. Glutathione Sepharose was purchased from Pharmacia (Milton Keynes, UK) and alkylated trypsin from Promega (Southampton, UK).

Buffer solutions:

Buffer A; 50 mM Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1 % (by vol) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β -glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μ M Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 10 μ g/ml leupeptin, 0.1 % (by vol) 2-mercaptoethanol.

Buffer B; 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.03 % (by vol) Brij-35, 0.27 M sucrose, 0.1 % (by vol) 2-mercaptoethanol.

Buffer C; 50 mM Tris/HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 50 mM NaF 0.1% (by vol) 2-mercaptoethanol, 0.1 mM PMSF, 1 mM benzamidine.

Expression of GST-PKB α in 293 cells. The DNA sequence encoding PKB α was subcloned into the eukaryotic expression vector PEBG2T that has been used to overexpress GST-fusion proteins in eukaryotic cells under an EF1 α promoter [27]. A PCR reaction was set up to generate a full length cDNA encoding the PKB α gene with a BamH1 site at the 5' end of the cDNA that is in frame with the GST sequence of the PEBG2T vector and the ATG initiation codon of PKB α , and a KpnI site at the 3' end, using the human PCMV5 -HA-PKB α plasmid [15]. The DNA sequence of the BamH1/KpnI cDNA fragment was checked and then subcloned into the unique BamH1/KpnI restriction sites on the pEBG 2T expression vector. In order to prepare GST-PKB α , 40, 10 cm diameter dishes of human embryonic kidney 293 cells were cultured and each dish transfected with 20 μ g of GST PKB α expression construct using the modified calcium phosphate method described previously for overexpression of haemagglutinin tagged PKB α in 293 cells [15]. 24 h after transfection, the cells were serum starved for 16 h and each dish of cells lysed in 1 ml of ice-cold Buffer A. The 40 lysates were pooled, centrifuged at 4°C for 10 min at 13, 000 x g and the supernatant incubated for 60 min on a rotating platform with 800 μ l of glutathione-Sepharose previously equilibrated in Buffer A. The suspension was centrifuged for 1 min at 3000 x g, the beads washed three times with 10 ml of Buffer A containing 0.5 M NaCl, and then a further 10 times with 10 ml of Buffer B to ensure complete removal of all the Triton X-100 which interferes with the activation of GST-PKB α by PDK1 (Fig 3B). GST-PKB α was eluted from the resin at ambient temperature with three 1 ml portions of Buffer B containing 20 mM glutathione pH 8.0. The combined eluates (0.65 mg/ml protein) were divided into aliquots, snap frozen in liquid nitrogen, and stored at -80°C.

Preparation of phospholipid vesicles

Phospholipid vesicles comprising 1 mM PtdCho, 1 mM PtdSer and 0.1 mM PtdIns lipids were prepared, dried to a film under vacuum and resuspended with vortexing into 10 mM Hepes, pH 7.3. The cloudy
5 solution of multilamellar and large unilamellar vesicles was bath-sonicated for 20 min, after which a clearer suspension of smaller unilamellar vesicles was obtained. Solutions were stored at 4°C at concentrations 10-fold higher than those required in the assay and used within 2-4 days.

10 Purification of PDK1 from rabbit Skeletal muscle

Day 1. A female New Zealand White rabbit was killed with a lethal dose of sodium pentobarbitone. Skeletal muscle from the hind limbs and back (500g) was excised rapidly and placed on ice. All subsequent steps were carried out at 0-4°C. The muscle was minced, homogenized in 2.5 vol
15 of 25 mM Tris/HCl pH 7.5, 4 mM EDTA, 2 mM EGTA, 50 mM NaF, 0.1% (by vol) 2-mercaptoethanol, 0.1 mM PMSF and 1 mM benzamidine and centrifuged for 30 min at 4200 x g. The supernatant was filtered through glass wool and poured through a Buchner funnel containing 10 g of QAE-Sephadex equilibrated in Buffer C. The column was washed with
20 one litre of Buffer C containing 50 mM NaCl, and washed with 700 ml of Buffer C containing 0.2 M NaCl to elute PDK1. A 50% (by mass) solution of polyethylene glycol (PEG) was added to the eluate to bring the final concentration of PEG to 4%. After stirring on ice for 60 min, the suspension was centrifuged for 15 min at 10 000 x g. The supernatant
25 was decanted and made 15% in PEG by further addition of 50% (by mass) PEG. After stirring for a further 60 min on ice, the suspension was again centrifuged for 15 min at 10 000 x g. The supernatant was discarded and the precipitate dissolved in 50 ml of ice cold Buffer B, filtered through a 0.25 µm filter and then chromatographed on an SP-Sepharose column (11
30 x 1.6 cm) equilibrated in Buffer B. The column was developed with a

300 ml linear gradient of NaCl to 0.5 M at a flow rate of 3 ml/min and 6 ml fractions were collected (Fig 2A). The active fractions were pooled and applied directly on to a 5 ml heparin-Sepharose column (HiTrap) equilibrated in Buffer B containing 0.1 M NaCl. The column was developed with a 90 ml linear salt gradient to 0.9 M NaCl at a flow rate of 2 ml/min and fractions of 1 ml were collected. PDK1 activity eluted as 2 peaks. Peak-1, comprising ~30% of the activity, was eluted at 0.5 M NaCl, while Peak 2 comprising ~70% of the activity, eluted at 0.7 M NaCl (Fig 2B).

Day 2. The active fractions of Peak 2 were pooled concentrated to 0.2 ml by ultrafiltration, diluted to 2.0 ml in Buffer B, and loaded on to a Mono S column (5 cm x 1.6 mm) equilibrated in Buffer B. The column was developed with a 4.0 ml linear gradient of salt to 0.15 M MgCl₂ at a flow rate of 0.2 ml/min and 0.1 ml fractions were collected. Fractions containing PDK1 were aliquoted, snap frozen in liquid nitrogen, and stored at -80°C. No significant loss of activity occurred upon thawing.

Assay of PDK1. The assay was carried out in two stages; in the first, GST-PKB α was incubated with PDK1 in the presence of MgATP and phospholipid vesicles to permit activation of GST-PKB α . In the second stage, the solution was made 0.5 % (by vol) in Triton X100 (which completely inhibits phosphorylation and activation of GST-PKB α without affecting GST-PKB α activity, see Fig 3B), together with Mg[γ -³²P]ATP, and the specific PKB α substrate peptide RPRTAAF [16].

In Stage 1, an 18 μ l reaction mixture was set up containing 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 0.1 mM EGTA, 2.5 μ M PKI, 1 μ M microcystin-LR, 10 mM Mg(Ac)₂, 100 μ M unlabelled ATP, 0.6 μ M GST-PKB α , 100 μ M PtdSer, 100 μ M

PtdCho in the presence or absence of 10 μ M PtdIns(3,4,5)P₃. The assay was initiated by the addition of 2 μ l of PDK1 and, after incubation for 30 min at 30°C, stage 2 of the assay was initiated by the addition of 30 μ l of a mixture containing 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1 % (by vol) 2-mercaptoethanol, 0.1 mM EGTA, 2.5 μ M PKI, 1 μ M microcystin-LR, 10 mM Mg(Ac)₂, 100 μ M [γ ³²P]ATP (200-400 cpm/pmol), 100 μ M of the peptide RPRTAAF and 1.25 % (by vol) Triton X-100. After 10 min at 30°C, the reactions were terminated by spotting the reaction mixture on to p81 phosphocellulose paper. The papers were washed in phosphoric acid and analysed as described previously [28]. A control reaction in which GST-PKB α was omitted was taken as the blank and was always less than 5% of the activity measured in the presence of GST-PKB α . The basal GST-PKB α activity is the activity measured in the absence of PDK1. One Unit of PDK1 activity was defined as that amount required to increase the basal activity of GST-PKB α by 1 Unit of activity. 1 Unit of GST-PKB α activity was that amount of enzyme required to catalyse the phosphorylation of 1 nmol of the peptide RPRTAAF in 1 min. The assays were linear with time up to a final concentration of 2 U/ml of PDK1 activity in the assay.

Phosphorylation of GST-PKB α by PDK1. The incubations were identical to the Stage 1 described above except that [γ ³²P] ATP (200-400 cpm/pmol) was used instead of unlabelled ATP. The reactions were terminated by making the solution 1 % (by mass) in SDS. The samples were run on a 7.5 % SDS-polyacrylamide gel and, after staining with Coomassie blue, the gels were autoradiographed. The Coomassie blue-staining bands corresponding to GST-PKB α were excised and the ³²P-radioactivity associated with each band was quantified.

Mapping the site on GST-PKB α phosphorylated by PDK1. GST-PKB α

was phosphorylated in the presence of the D-enantiomer of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P₃ except that the scale of the reaction was increased 10-fold and the specific activity of the [γ ³²P] ATP was increased to 1500 cpm/pmol. The reaction was stopped by the addition of SDS and
5 2-mercaptoethanol to final concentrations of 1% (by mass) and 1% (by vol) respectively, and heated for 5 min at 95°C. After cooling to ambient temperature, 4-vinylpyridine was added to a concentration of 2.5% (by vol) and the sample left on a shaking platform for 1 h at 30°C to alkylate cysteine residues. The sample was then electrophoresed on a 7.5% SDS
10 polyacrylamide gel, and the ³²P-labelled GST-PKB α eluted from the gel and digested with trypsin as described previously [15].

Results

15 **Purification of GST-PKB α from 293 cells.** PKB α was expressed in 293 cells as a fusion protein with glutathione S-transferase (GST) at the N-terminus, and purified on glutathione Sepharose. The preparation showed a major Coomassie blue-staining protein band of apparent molecular mass 85 kDa corresponding to GST-PKB α (Fig 1). The purity estimated by
20 densitometric analysis of the gels was >70% and 2 mg of purified GST-PKB α was obtained routinely in each preparation made from 40, 10 cm dishes of 293 cells. GST-PKB α from unstimulated 293 cells possessed very low activity, but was activated 20-fold and 45-fold after stimulation of 293 cells with insulin and IGF1, respectively (data not shown),
25 indicating that it is recognised by the upstream protein kinase(s) that activates PKB α *in vivo*. GST-PKB α from unstimulated 293 cells was therefore used as the substrate with which to identify its upstream activators.

30 **Identification of a PtdIns(3,4,5)P₃ dependent protein kinase that**

activates PKB α . We identified an activator of GST-PKB α in skeletal muscle cytosol that eluted from QAE-Sephadex at 0.25 M salt and purified it a further 30,000-fold from the QAE-Sephadex eluate (Table 1). The activator was completely dependent on PtdIns(3,4,5)P3 for activity and, because it phosphorylates PKB α (see below), it is hereafter termed PtdIns(3,4,5)P3-dependent protein kinase-1 (PDK1).

PDK1 could not be detected in the crude cytosol, but assuming a recovery of 50% through the initial batchwise chromatography on QAE-Sephadex, the overall purification was about 500,000-fold, and the entire preparation could be completed within 36 h. PDK1 was eluted as a single peak from S-Sepharose (Fig 2A), but resolved into two species on heparin-Sepharose. The minor component (Peak 1) eluted at 0.5M NaCl and the major component (Peak 2) at 0.7M NaCl (Fig 2B). All further experiments were carried out with the Peak 2 enzyme from heparin-Sepharose further purified through Mono S (Fig 2C), unless stated otherwise. After the final step the peak 2 enzyme, which was devoid of PKB activity, showed three major protein staining bands with apparent molecular masses of 85, 67 and 45 kDa. Only the 67 kDa band became phosphorylated upon incubation with MgATP and phosphorylation was greatly increased in the presence of PtdIns(3,4,5)P3. The sequences of two tryptic peptides from the 67 kDa band were highly homologous to regions of the catalytic domains of other protein kinases (data not shown). The data indicate that PDK1 is a novel 67 kDa protein kinase, distinct from other protein kinases reported to be activated by PtdIns(3,4,5)P3, such as PKC ϵ and PKC ζ .

Table 1. Purification of PDK1 from rabbit skeletal muscle.

500 g of muscle was used in this preparation. The protein was estimated by the procedure of Bradford [29]. PEG, polyethyleneglycol.

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
1. Extract	1400	17000	not measurable	-	-	-
2. QAE-Sephadex	750	555	1330	2.4	1	100
3. 4-15% PEG	55	164	1190	7.3	3	89
4. S-Sepharose	88	10	610	61	25	46
5. Heparin-Sepharose	11	0.01	290	29000	12000	22
6. Mono S	0.3	0.002	139	69500	29000	10

Phosphorylation and activation of GST-PKB α by PDK1. Purified PDK1 phosphorylated GST-PKB α in the presence of MgATP and phospholipid vesicles containing PtdCho, PtdSer and PtdIns(3,4,5)P3 to a level approaching 0.7 moles of ^{32}P /mol protein (Fig 3A).

5 Phosphorylation was paralleled by a >30-fold increase in activity (Fig 3A) which reached a specific activity of 80 U/mg. This is similar to the activity of PKB α which has been partially activated by mutating Thr-308 to Asp [15,16]. Omitting either PtdIns(3,4,5)P3 or PDK1 or ATP from the reaction abolished activation and phosphorylation of GST-PKB α (Fig 10 3B). Moreover, addition of 0.5% (by vol) Triton-X100 to the assays also prevented the activation and phosphorylation of GST-PKB α by PDK1 (Fig 3B), as did incubating PDK1 for 2 min at 55°C. In the absence of PtdCho/PtdSer vesicles, Ptd Ins(3,4,5)P3 was at least 15-fold less effective in activating PDK1 (Fig 3B).

15 When PDK1 was incubated for 30 min at 30°C in the presence of phospholipid vesicles containing PtdCho, PtdSer, PtdIns(3,4,5)P3 and MgATP, and then for 2 min at 55°C to inactivate PDK1, the phospholipid vesicles were unable to activate or phosphorylate GST-PKB α , unless more 20 PDK1 was added (data not shown). These observations establish that PDK1 does not activate GST-PKB α indirectly by converting PtdIns(3,4,5)P3 to another product that stimulates the autophosphorylation and autoactivation of GST-PKB α .

25 **Dependence of PDK1 on PtdIns(3,4,5)P3 concentration.** We next investigated the effect of varying the PtdIns(3,4,5)P3 concentration on the ability of PDK1 to activate (Fig 4A) and phosphorylate (Fig 4B) GST-PKB α . These experiments were carried out either by varying the concentration of PtdIns(3,4,5)P3 whilst maintaining the concentration of 30 both PtdCho and PtdSer at 100 μM or by maintaining a 10-fold excess of

PtdCho and PtdSer over PtdIns(3,4,5)P₃. Under both conditions, the concentration of PtdIns(3,4,5)P₃ required for half maximal activation or phosphorylation was 1-2 μ M, with an optimal effect at 10 μ M.

- 5 **PDK1 phosphorylates Thr-308 of PKB α .** In order to identify the residue(s) in PKB α phosphorylated by PDK1, ³²P-labelled GST-PKB α that had been phosphorylated to 0.4-0.6 mol/mol with PDK1, was digested with trypsin and chromatographed on a C18 column [15]. One major ³²P-labelled peptide was observed that eluted at 26% acetonitrile (Fig 5A).
- 10 This peptide, which coeluted with the ³²P-labelled tryptic peptide containing Thr-308 [15], was found to contain phosphothreonine only (data not shown). When this peptide was subjected to solid phase sequencing, ³²P-radioactivity was released after the first cycle of Edman degradation (Fig 5B) corresponding to Thr-308 [15]. Importantly, no ³²P-labelled
- 15 peptide eluted at the position corresponding to the tryptic peptide containing Ser-473, which elutes at 24% acetonitrile (Fig 5A, ref 15). These data establish that PDK1 only phosphorylates PKB α at Thr-308 *in vitro*.
- 20 **Lipid specificity of PDK1.** We next studied the ability of a panel of PtdIns derivatives to activate PDK1 when present in a vesicle background containing PtdCho/PtdSer. The predominant form of PtdIns(3,4,5)P₃ which occurs naturally is likely to be predominantly *sn*-1-stearoyl, 2-arachidonyl D-phosphatidylinositol 3,4,5-trisphosphate [17] (based on the
- 25 structure and fatty acid composition of natural PtdIns). Synthetic *sn*-1-stearoyl, 2-arachidonyl D-PtdIns(3,4,5,)P₃ (lipid 1 in Fig 6) proved highly effective, activating PDK1 activity 13-fold. By contrast, the L-enantiomer of this lipid induced only a 1.7-fold increase in PDK1 activity, which may be accounted for by trace contamination with the D-enantiomer. Whilst
- 30 the enantiomeric configuration of the head group was of critical

importance for activating PDK1, that of the glycerol moiety was not. Thus *sn*-2-arachidonyl, 3-stearoyl D- and L-PtdIns(3,4,5)P3 gave signals which were indistinguishable from the *sn*-1,2-derivatives (16-fold and 2.3-fold, respectively). The importance of unsaturated fatty acids in the diacylglycerol moiety is strongly indicated by the fact that *sn*-1,2-dilinoleoyl D-PtdIns(3,4,5)P3 (linoleic acid is C18:2) was the most effective lipid tested causing a 36-fold increase in GST-PKB α activity, whilst *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P3 induced only a 5.5-fold activation (palmitic acid is C16:0). In each of these experiments, the phosphorylation of GST-PKB α (Fig 6B) correlated with the extent of activation (Fig 6A).

Interestingly, *sn* -1,2-dipalmitoyl PtdIns(3,4)P2 (lipid 7, Fig 6) and *sn* -1,2-dipalmitoyl PtdIns(3,4,5)P3 (lipid 6, Fig 6) activated PDK1 to the same extent, both inositol phospholipids increasing GST-PKB α activity about 6-fold. However, PtdIns(3,5)P2 (lipid 8 Fig 6), PtdIns(4,5)P2 (lipid 9 Fig 6), PtdIns 4P (lipid 10, Fig 6), PtdIns 3P (lipid 11, Fig 6) and Ins(1,3,4,5)P4 did not activate PDK1 or induce the phosphorylation of GST-PKB α . In the absence of PDK1 none of the PtdIns derivatives tested induced any activation or phosphorylation of GST-PKB α (Fig 6).

Discussion

The identification and purification of PDK1 was greatly facilitated by the development of a specific peptide substrate (RPRAATF) for PKB α [16]. Other substrates used to assay PKB, such as histone H2B, myelin basic protein and Crosstide are phosphorylated by many protein kinases in cell extracts and obscured the detection of PDK1. The use of soluble GST-PKB α as a substrate was also important because immunoprecipitated HA-PKB was not phosphorylated effectively by PDK1. PDK1 was found to

phosphorylate PKB α at Thr 308 and to enhance its activity >30-fold. Since the phosphorylation of Thr-308 induced by insulin or IGF1 *in vivo* is prevented by inhibitors of PtdIns 3-kinase and PDK1 has an absolute requirement for PtdIns(3,4,5)P3 or PtdIns(3,4)P2, PDK1 is likely to be
 5 the enzyme which phosphorylates PKB α at Thr-308 *in vivo*. PDK1 activity is unaffected by wortmannin up to 2 μ M (data not shown), indicating that it is not a PtdIns 3-kinase family member.

The full activation of PKB α *in vitro* requires the phosphorylation of Ser-
 10 473 as well as Thr-308 [15]. Thus mutation of either Thr-308 or Ser-473 to Asp stimulates PKB α about 5-fold but, if both residues are mutated to Asp, activity is increased 18-fold. Similarly, phosphorylation of Ser-473 stimulates PKB α activity 7-fold, but if the phosphorylation of Ser-473 is combined with the mutation of Thr-308 to Asp, activity is increased 25-
 15 fold [15]. PKB α that has been partially activated by phosphorylation of Thr-308 or by the mutation of this residue to Asp does not become phosphorylated at Ser-473 *in vitro* upon incubation with MgATP in the presence of PtdIns(3,4,5)P3 ([15], Fig 5) indicating that Ser-473 is unlikely to be an autophosphorylation event, catalysed by PKB α itself.
 20 Ser-473 can be phosphorylated *in vitro* by MAP kinase-activated protein kinase-2, but this enzyme cannot mediate the insulin or IGF1-induced phosphorylation of PKB α at Ser-473 for reasons discussed in [15]. Since the insulin/IGF1-induced phosphorylation of Ser-473 is prevented by inhibitors of PtdIns 3-kinase, this residue may be phosphorylated by a
 25 distinct 3-phosphoinositide-dependent protein kinase (PDK2? - Fig 7). However, the Ser-473 kinase does not appear to be the PtdIns(3,4,5)P3-dependent peak-1 activity from heparin-Sepharose (Fig 2B), because this enzyme also phosphorylates PKB at Thr-308 (data not shown). The peak-1 may be a proteolytic fragment of peak-2 (or vice versa) or another
 30 isoform of PDK1. It is also possible that PtdIns(3,4,5)P3/PtdIns(3,4)P2

activates the Ser-473 kinase indirectly. For example, the phosphorylation of Ser-473 may be dependent on the binding of PtdIns(3,4,5)P3/PtdIns(3,4)P2 to the PH domain of PKB [18, 21] (but see below). Alternatively, the Ser-473 kinase may be activated by PDK1.

- 5 The mechanism by which insulin induces the activation of PKB α is shown schematically in Fig 7.

The activation of PDK1 by PtdIns(3,4,5)P3 is extremely specific, because only D-enantiomers of PtdIns(3,4,5)P3 are effective and many other
10 PtdIns phospholipids are inactive. Although the enantiomeric configuration of the glycerol moiety is not important, the presence of one or more unsaturated fatty acids greatly influences the extent of activation of PDK1 by PtdIns(3,4,5)P3 analogues (Fig 6). Since unsaturated fatty acids discourage tight packing of adjacent phospholipid molecules, it is
15 possible that this arrangement allows for more efficient interaction between membrane-inserted PtdIns(3,4,5)P3 and its effectors, perhaps explaining for the first time the biological significance of the unusual fatty acid composition of inositol phospholipids.

- 20 PtdIns(3,4,5)P3 and/or PtdIns(3,4)P2 have no effect on the activity of PKB α under conditions where these inositol phospholipids activate PDK1 strongly (Fig 6) consistent with our previous findings [18]. This observation, together with the finding that the activation of PKB α by insulin or IGF1 results from its phosphorylation at Thr-308 and Ser-473
25 [15], appears to exclude direct activation of PKB α by 3-phosphoinositides as a mechanism for its activation *in vivo*. Our results disagree with recent reports which have claimed that PKB α is activated directly by PtdIns(3,4)P2 [19-21]. Contamination of PKB α preparations with PDK1 activity may explain this discrepancy; recall that the activation of
30 phosphorylase kinase by cyclic AMP [22] was later shown to result from

contamination with a separate cyclic AMP-dependent protein kinase [23]. It has also been reported that PKB α is inhibited by PtdIns(3,4,5)P3 [20,21], but none of the four PtdIns(3,4,5)P3 derivatives we tested inhibited the basal PKB α activity at all, while all four were capable of
5 activating PDK1. It is possible that the synthetic PtdIns(3,4,5)P3 used in [20, 21] contains impurities that inhibit PKB α and or PDK1.

Although PKB is not activated directly by PtdIns(3,4,5)P3 or PtdIns(3,4)P2, it does bind these inositol phospholipids with micromolar
10 affinity [18, 21], via the N-terminal pleckstrin homology (PH) domain [18, 21]. In contrast, PKB binds PtdIns(4,5)P2 with a 10-fold lower affinity and does not bind to other inositol phospholipids tested [18, 21]. These findings raise several interesting possibilities. Firstly, the interaction of PtdIns(3,4,5)P3 and/or PtdIns(3,4)P2 with PKB may alter
15 its conformation in such a way as to facilitate phosphorylation by PDK1 and the Ser-473 kinase. Secondly, the formation of PtdIns(3,4,5)P3 and/or PtdIns(3,4)P2 in the plasma membrane may recruit PKB to this membrane also facilitating its activation by PDK1 and the Ser-473 kinase. However, neither of these mechanisms appear to be essential for the
20 activation of PKB *in vivo*, because a mutant lacking the PH domain can still be activated at least as well as wild-type PKB in response to insulin [24, 25]. Alternatively, recruitment of PKB to the plasma membrane could be a mechanism for facilitating the phosphorylation of membrane-bound PKB substrates. However, it should be noted that we purified
25 PDK1 from the cytosol of skeletal muscle. It will clearly be important to examine whether PDK1 localises to the plasma membrane when cells are stimulated with agonists which trigger a rise in the concentration of PtdIns(3,4,5)P3 and PtdIns(3,4)P2.

Example 2: Cloning of PDK1

We obtained the amino acid sequences of 9 peptide derived from PDK1 that we purified from rabbit skeletal muscle, these were:

5 LDHPFFVK;
ANSFVGTAQYVSPELL;
AGNEYLIFQK;
AHPFFESVTWENLHQQTTPK;
10 SGSNIEQYIHDLDNSFELDL;
QAGGNPWHQFVENNLILK;
QLLLTEGPHLYYVDPVNK;
TFFVHTPNR;
YQSHPDAAVQ;

15 We then put these sequences through the BLAST ncbi database search on the Internet and we discovered that all of these 9 peptide sequences were encoded by 2 EST sequences. The information regarding these EST sequences available from the Internet is given in Figures 8 and 9. We
20 then requested the ESTs from the UK HGMP Resource Centre, I.M.A.G.E. Consortium, Hinxton, Cambridge CB1 1SB, UK (a consortium part funded by the Medical Research Council).

We then determined the nucleotide sequences of the EST clones ourselves
25 by sequencing both DNA strands of the ESTs. There were a number of errors in the EST sequences available on the Internet. From the data we obtained it is clear that the EST sequences represent two overlapping clones of PDK1.

30 Most of the open reading frame of PDK1 was derived by interrogation of

the dbest database at the National Centre for Biological Information. A full length cDNA clone for PDK1 was isolated by hybridisation screening of a cDNA library, in the vector λ ZAP, made from the human breast cancer cell line MCF7 (a gift of P. Mitchell, Institute of Cancer Research, Sutton, UK). The PDK1 probe for the screening was generated by RT-PCR with the primers CTGAGCCAGTTTGGCTGC and ACGTCCTGTTAGGCGTGTGG corresponding to nucleotide 1138-1567 of the PDK1 sequence, with MCF7 cDNA as template. DNA sequencing was carried out on an Applied Biosystems 373A DNA automatic sequencer using the Taq dye terminator cycle sequencing kit.

The nucleotide and amino acid sequences for PDK1 are shown in Figure 10. The sequence encodes a 556 residue protein with a predicted molecular mass of 63.1 kDa. We expressed PDK1 protein with a Myc epitope Tag in 293 cells (see Examples below) and it migrates as a 68-70 kDa band, and PDK1 antibodies we have raised recognise a 69 kDa band in 293 cell lysates (data not shown). For this reason, and because the initiating methionine lies in a good Kozak consensus sequence, the sequence shown in Figure 10 is likely to represent the whole of PDK1.

Polynucleotides containing a full length coding sequence for PDK1 are readily obtained by, for example, using suitable PCR primers based on the nucleotide sequence given in Figure 10 and amplifying the PDK1 cDNA from a suitable cDNA library or from reverse-transcribed mRNA.

Also a polynucleotide containing a coding sequence for PDK1 is obtained by ligating appropriate portions of IMAGE clone 626511 and IMAGE clone 526583 following digestion with suitable restriction endonucleases or by using PCR-based strategies for joining appropriate aparts of IMAGE clone 526583 and IMAGE clone 626511.

It will be appreciated that a full length coding sequence for PDK1 can readily be obtained from a suitable cDNA library using the sequence information given in Figure 10 to generate a probe and that the sequence of any clone obtained in this way can be determined using the Sanger dideoxy sequencing method.

The human ESTs encoding PDK1 were isolated from many different human tissues (Table A below) indicating that PDK1 is expressed ubiquitously. Interestingly, the nucleotide sequence of PDK1 possessed 100% identity to the partial sequence of a gene that has been mapped to a 700-kb region on Human Chromosome 16p13.3 close to the genes responsible for polycystic kidney disease type 1 and tuberous sclerosis type 2 disease [23]. This gene is known to be expressed as a large 8-kb transcript in heart, brain, placenta, lung, skeletal muscle, kidney and pancreas [23].

Table A. List of overlapping Expressed Sequence Tags in the database encoding for PDK1 gene. Accession numbers are from gene bank. The tissue is the tissue from which the EST was derived from.

Accession Number	Tissue
AA186323	HeLa cells
H97903	melanocyte
AA018098	retina
AA018097	retina
AA019394	retina
AA019393	retina
N22904	melanocyte
W94736	fetal heart
EST51985	gall bladder

	N31292	melanocyte
	AA188174	HeLa cells
	AA100210	colon
	R84271	retina
5	AA121994	pancreas

Example 3: Alternative cloning strategy for PDK1

The 67 kDa band corresponding to PDK1 was cleaved with trypsin and
 10 the digest chromatographed on a Vydac C18 column to resolve the tryptic
 peptides. The sequences of the following three peptides were established:

1. **ANSFVGTAQYVSPELL**
2. AGNEYLIFQK
- 15 3. LDHPFFVK

These peptides are homologous to sequences found in the catalytic
 domains of other protein kinases. Peptide 1 is homologous to a region of
 subdomain VIII, peptide 2 to a region of subdomain X and peptide 3 to a
 20 region of subdomain XI. These sequences are used to clone PDK1 using
 standard PCR-based strategies.

Polymerase chain reactions (PCR) are carried out using the following
 oligonucleotides coding the peptides 1 and 3 (or slight variations thereof;
 25 for example the position I may be inosine or various combinations of G,
 C, A and T):

5'TTTGT(G/T)GGIACIGCICA(A/G)TA(T/C)GT 3'

30 coding for the part of peptide 1 shown in boldface type.

5' TTLAC(A/G)AA(A/G)AAIGG(A/G)TG(A/G)TC 3'

coding for peptide 3.

- 5 The resulting PCR fragment contains the region coding for peptide 2 and is analysed by Southern blotting and hybridisation with the following oligonucleotide coding for peptide 2 (or slight variations thereof: for example the position I may be inosine or various combinations of G, C, A and T):

10

5' AA(T/C)GA(A/G)TA(T/C)(C/T)TIAT(T/C/A)TT(TC)CA(A/G)AA3'

Positive PCR fragments are subcloned and used to screen skeletal muscle and other cDNA libraries to isolate a full length clone for PDK-1.

15

The full-length clone for PDK-1 is sequenced using the Sanger dideoxy method.

20

The rabbit PDK-1 cDNA may be obtained first and this is used to screen a human skeletal muscle cDNA library to isolate a full-length human PDK-1 cDNA. Molecular biology techniques used are essentially as described in Sambrook *et al* (1989) Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

25

Example 4: Further alternative cloning strategy for PDK1

Antibodies are raised against PDK1 purified as described in Example 1.

30

The antibodies are used to screen a λ gt11 expression library made from

cDNA copied from human or rabbit skeletal muscle mRNA.

Positive clones are identified and the insert sequenced by the Sanger method as in Example 2.

5

Example 5: Expression of human PDK1 and activation of PKB α ; homology to *Drosophila* DSTPK61

In order to elucidate the role of PDK1 *in vivo* and to determine the
10 mechanism by which it is regulated by PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂
it was clearly useful to first determine the structure of this enzyme and the
mechanism by which PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ stimulates PDK1 to
phosphorylate PKB. In this and previous Examples, we describe the
cloning, sequencing and expression of human PDK1, and demonstrate that
15 the expressed enzyme activates PKB α *in vitro* in an identical manner to
PDK1 purified from muscle. PDK1 also activates PKB α in cotransfection
experiments and it also potentiated the phosphorylation of Thr308 induced
by IGF1. Surprisingly, PDK1 was found to be structurally and
functionally homologous to the *Drosophila* protein kinase DSTPK61,
20 which has been implicated in the regulation of sexual development in
Drosophila.

Materials and Methods

25 **Materials**

All phospholipids were obtained from the sources described previously
[21]. Glutathione Sepharose, the pGEX-3X expression vector and 1 ml
heparin-Sepharose column (HiTrap) were purchased from Pharmacia
30 (Milton Keynes, UK); monoclonal antibodies 12CA5 and 9E10 were from

Boehringer Mannheim (Lewes, UK) and alkylated trypsin and the pSP72 cloning vector from Promega (Southampton, UK). GST-PKB α and sources of all other materials are described in [21]. The pCR 2.1-TOPO cloning vector was from Invitrogen (Leek, Netherlands).

5

Buffer solutions. Buffer A: 50 mM Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β -glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μ M microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 10 μ g/ml leupeptin, 0.1% (by vol) 2-mercaptoethanol. Buffer B: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.03% (by vol) Brij-35, 0.27 M sucrose, 0.1% (by vol) 2-mercaptoethanol.

10

15

Preparation of DNA expression constructs encoding GST-PDK1, Myc-PDK1. Two overlapping human ESTs encoding PDK1 (GenBank accession numbers AA121994 and AA186323 corresponding to nucleotides 98 to 708 and 467 to 1811 of PDK1 (Fig 1) were obtained from the I.M.A.G.E. consortium [42] and sequenced. The two sequences were

20

joined together by an *ScaI* restriction enzyme digest of each EST clone, and the appropriate restriction fragments obtained from these digests were ligated to generate a plasmid containing a continuous PDK1 sequence from nucleotides 154 to 1670. This construct was used as a template for a PCR reaction to generate an N-terminal epitope-tagged Myc-PDK1 (amino acid

25

residues 52-556) construct. This was achieved using the oligonucleotides GCGGAGATCTGCCACCATGGAGCAGAAGCTGATCTCTGAAGAGGACTTGGACGGCACTGCAGCCGAGCCTCGG and GCGGGGTACCTCACTGCACAGCGGCGTCCGGGTG that incorporate a *Bgl*III site (underlined) and a *Kpn*I site (double underlined). The

30

resulting PCR fragment was subcloned into the *Bgl*III/*Kpn*I sites of an

pSP72 cloning vector, and the nucleotide sequence confirmed by DNA sequencing. The Myc-PDK1 coding sequence was subcloned from this vector as a *Bgl*II-*Kpn*I fragment into the *Bam*HI and *Kpn*I sites of the eukaryotic expression vectors pEBG2T [43] in order to generate a construct for the expression of GST-PDK1 in 293 cells. The same fragment was ligated into the *Bgl*II and *Sa*I sites of the vector pCMV5 [44] to generate a construct for the expression of Myc-PDK1 in 293 cells. The structure of each construct was verified by DNA sequencing, and plasmid DNA for transfection was purified using the Qiagen plasmid Mega kit according to the manufacturer's protocol.

N-terminal Myc epitope tagged PDK1 constructs encoding the full length protein (residues 1 to 556) and lacking the PH domain (residues 1 to 450) were generated by a PCR approach using the full length full length PDK1 cDNA isolated from the MCF7 library. This was achieved by using the 5' primer

gcggagatctgccaccatggagcagaagctgatctctgaagaggacttggccaggaccaccagccagc
tgtatgacg (for both the full length and Δ PH-PDK1 constructs) and the 3' primers gcggggtacctcactgcacagcggcgctccgggtg (for full length PDK1) and gcggggtacctcagtgccaaagggtttccgccagcctgctt (for the Δ PH-PDK1 construct).

The resulting PCR fragments were cloned into the pCR 2.1-TOPO vector and subsequently subcloned into the pEGB-2T vector as *Bgl*II-*Kpn*I fragment and pCMV5 as an *Eco*RI-*Kpn*I fragment. A full length catalytically inactive PDK1 construct in which Asp223 was changed to Ala was created by the PCR-based megaprimer strategy (Tao & Lee (1994) In: Griffin & Griffin (eds) PCR Technology: Current Innovations, CRC Press, Boca Raton, Florida, pp 69-83) and then subcloned into pCMV5 and pEBG-2T as described above. The structure of each construct was verified by DNA sequencing, and plasmid DNA for transfection was purified using the Qiagen plasmid Mega kit according to the

manufacturer's protocol.

A bacterial expression vector for GST-DSTPK61 was prepared by ligating a *EcoRI/EcoRV* fragment of the *Dstpk61* cDNA derived from pBluescriptSK into the *EcoRI/SalI* sites of the pGEX-3X expression vector.

Preparation of DNA expression constructs encoding GST- Δ PH-PKB α .

A PCR based strategy was used to prepare a GST- Δ PH-PKB α (encoding residues 118-479 PKB α) construct using as a template a full length human PKB α construct that was isolated from a human skeletal muscle cDNA library and subcloned into the pBluescriptSK vector. The GST- Δ PH-PKB α construct was obtained using the 5' primer cgggatccatggacttccggctcgggctca and 3' primer was the T7 oligonucleotide of pBluescriptSK vector. The resulting PCR fragment was cloned into pBluescriptSK as a *BamHI-KpnI* fragment, and subsequently into pEBG-2T as a *BamHI-KpnI* fragment.

Expression of GST-PDK1, GST- Δ PH-PDK1, GST-D223A-PDK1, GST-

Δ PH-PKB α and GST-DSTPK61. Twenty 10 cm diameter dishes of human embryonic kidney 293 cells were cultured and each dish transfected with 20 μ g of DNA encoding either GST-PDK1 (residues 52-556), GST-PDK1 (residues 1-556), GST- Δ PH-PDK1, GST-D223A-PDK1, or GST- Δ PH-PKB α using a modified calcium phosphate method [20]. 24 h after transfection, the cells were serum starved for 16 h and each dish of cells lysed in 1 ml of ice-cold Buffer A. The 20 lysates were pooled, centrifuged at 4°C for 10 min at 13,000 x g and the supernatant incubated for 60 min on a rotating platform with 1 ml of glutathione-Sepharose previously equilibrated in Buffer A. The suspension was centrifuged for 1 min at 3000 x g, the beads washed three times with 10 ml of Buffer A

containing 0.5 M NaCl, and then a further 10 times with 10 ml of Buffer B to ensure complete removal of all the Triton X-100 which interferes with the activation of GST-PKB α by PDK1 [21]. GST-PDK1 α was eluted from the resin at ambient temperature with three 1 ml portions of Buffer B containing 20 mM glutathione pH 8.0. The combined eluates (0.8 mg/ml protein) were divided into aliquots, snap frozen in liquid nitrogen, and stored at -80°C. Between 0.5 and 2.0 mg of each GST-fusion protein was obtained and was more than 90% homogeneous as judged by SDS polyacrylamide gel electrophoresis.

E. coli transformed with an expression plasmid encoding GST-DSTPK61 were grown at 37°C in LB medium to an absorbance of 0.6 at 600 nm. Isopropyl- β -D-thiopyranoside was added to 30 μ M and the bacteria were incubated for 16 h at 25°C before centrifuging for 10 min at 4000 x g.

The bacteria were resuspended in 15 ml of Buffer A and lysed by sonication for 4 min on ice. The suspension was centrifuged for 30 min at 30 000 x g and the GST-DSTPK61 was purified by affinity chromatography on 1 ml of glutathione-Sepharose as described above for GST-PDK1. The GST-DSTPK61 derived from this (0.35 mg/ml) was divided into aliquots, snap frozen in liquid nitrogen and stored at -80°C.

Assay of GST-PDK1 and GST-DSTPK61 activity. GST-PDK1 and GST-DSTPK61 were diluted appropriately in Buffer B containing 1 mg/ml bovine serum albumin, and assayed for their ability to activate and phosphorylate GST-PKB α [21]. One unit of PDK1 activity was defined as that amount required to increase the basal activity of GST-PKB α by 1 unit of activity in one min. One unit of GST-PKB α activity was that amount of enzyme required to catalyse the phosphorylation of 1 nmol of the peptide RPRAATF in 1 min in an assay containing 0.1 mM RPRAATF [45]). In order to ensure that the assay was linear with respect

to time the concentration of GST-PDK1 or GST-DSTPK61 was below 2 U/ml. At this concentration the level of phosphorylation of PKB α was <0.4 mol phosphate per mol protein in the 30 min assay.

5 **Transfection of 293 cells with HA-tagged PKB α and Myc-PDK1.**

Human embryonic kidney 293 cells were cultured on 10 cm diameter dishes and transfected with 2 μ g/ml pCMV5 DNA constructs encoding for HA-PKB α or HA-PKB α plus Myc-PDK1 [20]. After 24 h the cells were deprived of serum for a further 16 h and then stimulated for 10 min with
10 either 100 ng/ml IGF1 or Buffer. The cells were lysed in 1.0 ml of ice-cold Buffer A, the lysate centrifuged at 4°C for 10 min at 13,000 x g and HA-PKB α immunoprecipitated from aliquots of the supernatant (10 μ g protein) [20] and assayed for PKB α with the peptide RPRAATF [45] as described previously [20].

15

³²P-labelling of 293 cells transfected with HA-PKB α or Myc-PDK1.

This was carried out essentially as described previously [20]. Briefly, 293 cells were transfected with HA-PKB α , or with HA-PKB α plus Myc-PDK1, washed with phosphate free DMEM, incubated for 4 h with [³²P]-
20 orthophosphate (1 mCi/ml), then stimulated with IGF1 (50 ng/ml) for 5 min. The cells were lysed, and the HA-PKB α immunoprecipitated (using 10 μ g of 12CAS antibody per 10 cm dish of cells) or the Myc-PDK1 immunoprecipitated (using 10 μ g of 9E10 antibody per 10 cm dish of cells). The immunoprecipitated protein was alkylated with 4-
25 vinylpyridine, subjected to SDS/polyacrylamide gel electrophoresis, digested with trypsin and analysed by chromatography on a C₁₈ column (Alessi *et al* (1996) *EMBO J.* **15**, 6541-6551).

Results

PDK1 is a human homologue of the *Drosophila* DSTPK61 protein kinase. The catalytic domain of PDK1 spans residues 83-342 (Fig 10) and contains all of the classical kinase subdomains I-XI [24]. It is most similar to the subfamily of protein kinases that include PKA (39% identity to the catalytic domain), PKC (35% identity to the ζ isoform) and PKB (35% identity). The PDK1 catalytic domain was even more similar to two yeast protein kinases of unknown function. The catalytic domain was 53% identical to the *S. pombe* protein kinase with GenBank accession number 1431588 and 48% identical to the *S. cerevisiae* protein kinase with accession number 1078290. However, there was no homology between PDK1 and these yeast protein kinases outside of the catalytic domain. In contrast, the *Drosophila* protein kinase named *Drosophila* Serine/Threonine Protein Kinase-61 (DSTPK61, accession number Y07908 ref 22) was not only 54% identical to PDK1 in the catalytic domain, but was very similar in the non catalytic C-terminal domain (Fig 11). This homology was most striking between residues 450 and 550 of PDK1 where the identity to DSTPK61 was 61% (79% similarity). These observations suggested that DSTPK61 might be a *Drosophila* homologue of PDK1. However, DSTPK61 contains a 60 residue C-terminal C-terminal extension not found in PDK1 and there is little homology between the N-terminal 70 residues of PDK1 and N-terminal 150 residues of DSTPK61 (Fig 11). PDK1 also lacks the polyglutamine insertion sequence present near the N-terminus of DSTPK61 (residues 120-150) and a further polyacidic insertion located between subdomains VII and VIII (residues 312 to 370) of the kinase domain (Fig 11). The physiological role of DSTPK61 will be considered further under Discussion.

PDK1 and DSTPK61 possess a C-terminal Pleckstrin Homology

domain. The high level of sequence conservation between PDK1 and STK61, from residues 450-550 of PDK1 (Fig 11), suggested that this region is likely to have an important function. Inspection of these sequences indicated that these are likely to form part of a Pleckstrin Homology (PH) domain. These domains of ~100 residues are found in over 70 other proteins and are predicted to fold into a similar 3-dimensional structures and may mediate protein-lipid, protein-protein interactions, or both [25,26]. We have performed sequence alignment of residues 450-550 in both PDK1 and STK61, together with sequences from PH domains of determined tertiary structure, pleckstrin, spectrin, dynamin, and phospholipase C- δ and also with 71 other known PH domain sequences (data not shown). Although the percentage identity is poor between PH domains in general there are certain positions that show high levels of residue type conservation. For PDK1 and DSTPK a single position (Tryptophan, position 113, position Trp-535 of PDK1) shows complete identity throughout the domain family, however there are also many similar amino acids at defined regions of the PH domain) (Figure 17). Secondary structure predictions also indicated that residues 450-530 of PDK1 (positions 1-80) are likely to contain regions of β -sheet, while the residues between 531-550 (positions 80-100) are likely to form an extended α -helix, a prediction that is consistent with the known structures of other PH domains [25,26].

Expression of GST-PDK1 and GST-DSTPK61. Residues 52-556 of PDK1 were expressed in human embryonic kidney 293 cells and residues 1-752 of DSTPK61 in *E. coli* as fusion proteins with glutathione S-transferase (GST) at the N-terminus (hereafter termed GST-PDK1 and GST-DSTPK61), and both were purified on glutathione-Sepharose. The GST-PDK1 preparation showed two bands with apparent molecular masses of 87 and 85 kDa (Figure 18, Lane 1) and 2 mg of GST-PDK1 purified

protein was obtained from 20 (10 cm diameter) dishes of cells. The GST-DSTPK61 preparation showed a diffuse band at the expected molecular mass (105 kDa) together with a number of minor degradation products (Figure 18, Lane 2). 0.35 mg of GST-DSTPK61 were obtained from 0.5
 5 litres of bacterial culture.

GST-PDK1 and GST-DSTPK61 activate and phosphorylate PKB α in a PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ dependent manner. GST-PDK1 and GST-DSTPK61 both activated and phosphorylated GST-PKB α in the
 10 presence of a vesicle background containing phosphatidylcholine (PtdCho) and phosphatidylserine (PtdSer) provided that PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ were included. The extent of activation of GST-PKB α correlated with the extent of phosphorylation (Fig 12), and no activation or phosphorylation occurred if PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ were
 15 replaced by either PtdIns(4,5)P₂ or PtdIns 3-P (Fig 12). Identical results were obtained using purified PDK1 from rabbit skeletal muscle and full length GST-PDK1 (residues 1-556) expressed in 293 cells ([21] and data not shown). A catalytically inactive GST-PDK1 mutant in which Asp223 was changed to Ala did not phosphorylate or active GST-PKB α in the
 20 presence of PtdCho/PtdSer lipid vesicles containing PtdIns(3,4,5)P₃ (data not shown). The specific activities of GST-PDK1 (residues 52 to 556, 78 000 U/mg) and GST-PDK1 (residues 1 to 556, 89 000 U/mg) towards GST-PKB α in the presence of PtdIns(3,4,5)P₃ were similar to PDK1 purified from rabbit skeletal muscle (100 000 U/mg), being over 200
 25 times higher than that of bacterially expressed GST-DST PK61 (280 U/mg). Further work is needed to establish whether this difference is caused by misfolding of DSTPK61 or lack of an important post-translational modification when it is expressed in *E. coli*, or whether DSTPK61 does not recognise human PKB α as well as human PDK1.

Some activation and phosphorylation of GST-PKB α was obtained using very high concentrations of GST-PDK1 or GST-DSTPK61 in the absence of PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂. However, the activity of PDK1 was enhanced about 1000-fold in the presence of these inositol phospholipids.

5 In the presence of PtdIns(3,4,5)P₃ lipids and at high GST-PDK1 and GST-DSTPK61 concentrations, the phosphorylation of GST-PKB α reached 0.75 mol phosphate per mol protein and was paralleled by a 35 fold increase in activity. This was similar to the maximal activation of GST-PKB α obtained using PDK1 from rabbit skeletal muscle [21].

10 **Expressed PDK1 and DSTPK61 phosphorylate PKB α at Thr308.** ³²P-labelled GST-PKB α that had been maximally phosphorylated with either GST-PDK1 (Fig 13A) or GST-DSTPK61 (Fig 13B), was digested with trypsin and chromatographed on a C₁₈ column. One major ³²P-labelled
15 peptide was obtained in each case. This peptide eluted at 25 % acetonitrile at the same position as the ³²P-labelled tryptic phosphopeptide containing Thr308 [20,21], and contained phosphothreonine and, when subjected to solid phase sequencing, ³²P-radioactivity was released after the first cycle of Edman degradation, confirming that this peptide is indeed that of PKB α
20 phosphorylated at Thr308 (data not shown).

PKB α is activated by cotransfection with PDK1 in 293 cells and phosphorylated at Thr308. In order to determine if PDK1 was capable of activating PKB α in a cellular context, we transfected a haemagglutinin
25 epitope-tagged PKB α (HA-PKB α) into 293 cells, either alone or together with Myc-epitope tagged PDK1 (Myc-PDK1). As reported previously, HA-PKB α possessed a low basal activity when transfected alone into 293 cells, which was increased 40-fold after stimulation with IGF1 (Fig 14, [20]). However, when 293 cells were transfected with both HA-PKB α
30 and Myc-PDK1, the activity of HA-PKB α was increased 20-fold in

unstimulated cells, and 70-fold after stimulation with IGF-1 (Fig 14). When 293 cells were transfected with HA-PKB α and a catalytically inactive mutant of PDK1 (Myc-D223A-PDK1), HA-PKB α was not activated significantly (Figure 14).

5

In order to establish the mechanism by which overexpression of PDK1 in 293 cells induced the activation of PKB α , the cells were incubated with 32 P-phosphate, stimulated with buffer or IGF1 and 32 P-labelled HA-PKB α was immunoprecipitated from the lysates. After digestion with trypsin,
 10 the resulting peptides were analysed by C₁₈ chromatography (Fig 15). As observed previously HA-PKB α is phosphorylated at Ser-124 and Thr-450 in unstimulated cells, and IGF1 stimulation induces the phosphorylation of Thr308 and Ser473 (Fig 15A and 15C). In contrast, when cotransfected with PDK1, HA-PKB α became partially phosphorylated at
 15 Thr308 in unstimulated 293 cells, to a level that was 70% of that observed in IGF1-stimulated cells transfected with HA-PKB α alone. This phosphorylation of Thr308 was increased a further 4-fold in response to IGF1 (Fig 15). Importantly, cotransfection of Myc-PDK1 with HA-PKB α did not induce phosphorylation of HA-PKB α at Ser473 in unstimulated
 20 cells, nor did it potentiate the level of Ser473 phosphorylation following IGF1 stimulation (Fig 15).

Role of the PH domain in the activation of PKB α by PDK1. A mutant GST-PKB α mutant lacking the PH domain (GST- Δ PH-PKB α , residues
 25 118-479) possessed a 3-fold higher basal activity than that of full length wild-type GST-PKB α was activated (Fig 16A) and phosphorylated (data not shown) by PDK1 in a PtdIns(3,4,5)P3 independent manner. However, the rate of activation was reduced about 20-fold compared to wild-type GST-PKB α (Fig 16A). PDK1 purified from rabbit skeletal muscle
 30 extracts also activated and phosphorylated GST- Δ PH-PKB α in a

PtdIns(3,4,5)P3 independent manner (data not shown).

A PDK1 mutant lacking the putative C-terminal PH domain was expressed as a GST-fusion protein in 293 cells (GST- Δ PH-PDK1, residues 1-450).

5 We found that this form of PDK1 was still able to activate GST-PKB α in a PtdIns(3,4,5)P3-dependent manner, but the rate of activation was reduced about 30-fold compared to full length wild-type GST-PDK1 (Fig 16B). As observed with PDK1 purified from rabbit skeletal muscle (see Example 1) or full length GST-PDK1 (data not shown), GST- Δ PH-PDK1
10 was activated more effectively by the D-enantiomer of *sn*-1-stearoyl, 2-arachidonoyl PtdIns(3,4,5)P3 than by the D-enantiomer of *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P3, and no activation was induced by the L-enantiomer of *sn*-1-stearoyl, 2-arachidonoyl PtdIns(3,4,5)P3 (Fig 9B). In contrast *sn*-1,2-dipalmitoyl D-PtdIns(3,4)P2 was very poor at inducing the
15 activation of GST-PKB α by GST- Δ PH-PDK1 (Fig 9B) under conditions where this lipid was as effective as *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P3 at stimulating GST-PKB α activation by either full length GST-PDK1 (data not shown) or PDK1 purified from rabbit skeletal muscle (see Example 1).

20 PDK1 is not activated or phosphorylated by IGF1. 293 cells were serum starved for 16h, stimulated with IGF1 lysed and the endogenous PDK1 activity present in the cell lysates was determined after chromatography on heparin-Sepharose (see Methods). IGF1 stimulation of cells for up to 10 min did not result in any activation or inhibition of
25 PDK1 activity (data not shown).

In order to see if IGF1 stimulation was inducing the phosphorylation of PDK1, 293 cells were transfected with Myc-PDK1 (encoding residues 52 to 556), incubated with 32 P-phosphate, stimulated with buffer or IGF1 for
30 5 min and 32 P-labelled Myc-PDK1 was immunoprecipitated from the

lysates. After digestion with trypsin, the resulting peptides were analysed by C₁₈ chromatography. These experiments demonstrate that PDK1 is phosphorylated at four tryptic peptides (only on serine residues) in unstimulated cells and that IGF1 does not alter the phosphorylation of any of these peptides (data not shown). Treatment of PDK1 purified from rabbit skeletal muscle with high concentrations of the serine/threonine-specific protein phosphatase 2A and protein tyrosine phosphatase 1B also had no effect on activity (data not shown). One of the *in vivo* phosphorylation sites on PDK1 was identified as Ser-241 which lies in the equivalent position to Thr308 of PKB in the kinase domain.

Discussion

PDK1 purified 500,000-fold from skeletal muscle [21] contains three proteins with apparent molecular masses of 85 kDa, 67-69 kDa and 45 kDa, and in this Example we established that the 67-69 kDa component is PDK1, and is likely to be a monomeric protein since it migrates with an apparent molecular mass of 70 kDa on gel filtration (data not shown). When cloned and expressed in 293 cells, this protein activated PKB α and induced its phosphorylation at Thr308, activation and phosphorylation both being dependent on PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂. PDK1 also activated PKB α in cotransfection experiments and potentiated the phosphorylation of Thr308 by IGF1. These observations and the finding that, like PKB, the mRNA encoding PDK1 is expressed in every tissue examined (Table A) are consistent with the notion that PDK1 lies "upstream" of PKB *in vivo*.

PKB α becomes phosphorylated at Ser473 *in vivo*, as well as at Thr308, in response to insulin or IGF1. Moreover the phosphorylation of Ser473 is essential for the full activation of PKB α and, like the phosphorylation

of Thr308, is prevented by wortmannin [20]. Importantly, PKB α did not become phosphorylated at Ser473 when cotransfected with PDK1, and nor did transfection with PDK1 affect the level of Ser473 phosphorylation after stimulation by IGF1. These experiments demonstrate that the protein kinase which phosphorylates PKB α at Ser473 is not activated by PDK1, that phosphorylation of T308 of PKB α in a cell does not cause PKB α to autophosphorylate at Ser-473, and that the requirement for PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ to trigger the phosphorylation of Ser473 is conferred by a different mechanism.

10

It is of interest that PDK1 lies in the same subfamily of protein kinase as PKB. Moreover, like PKB, it also contains a PH domain although, in contrast to PKB, this is located C-terminal to the catalytic domain [13]. The PH domain in PKB is capable of binding PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ at micromolar concentrations [27,28], which may facilitate its translocation to the plasma membrane that occurs in response to IGF1 [29] or interleukin-2 stimulation of EL4-IL-2 cells [30] to form a signalling complex. However, the finding presented in this paper that PDK1 is not activated by IGF1, and that a mutant of PKB α lacking the PH domain is activated and phosphorylated independently of PtdIns(3,4,5)P₃ (Fig 16A), demonstrates that the PtdIns(3,4,5)P₃-induced activation of PKB α by PDK1 is substrate directed, at least in part. It is possible that the binding of PtdIns(3,4,5)P₃ to the PH domain of PKB α , alters the conformation of PKB α so that Thr308 becomes accessible for phosphorylation by PDK1. Consistent with this model, deletion of the PH domain of PDK1 resulted in an enzyme that is still able to activate and phosphorylate PKB α in a PtdIns(3,4,5)P₃ dependent manner (Fig 16B).

However, the rate of activation of PKB α by PDK1 is reduced 30-fold when the PH domain of PDK1 is deleted. Thus it remains possible that

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the PH domain of PDK1 binds PtdIns(3,4,5)P3 and that this greatly enhances the rate of activation of PKB α . PtdIns(3,4,5)P3 can only stimulate the activation of PKB α by PDK1 when it is presented in lipid vesicles containing phosphatidyl choline and phosphatidyl serine (Example 1), and a PDK1 mutant lacking the PH domain may not be able to interact with lipids and hence be unable to penetrate these vesicles efficiently. This may account for the greatly reduced rate at which PDK1 lacking the PH domain activates PKB α . PKB α lacking the PH domain is also presumably unable to penetrate lipid vesicles and this may explain why this mutant is phosphorylated at a 20-fold lower rate by GST-PDK1 than full length PKB α (Fig 16A).

Although PKB α is activated by cotransfection with PDK1 in 293 cells, the overexpression of PDK1 does not potentiate the activation of PKB α by IGF1 (Fig 14). The reason is that the activation of PKB α also requires phosphorylation of Ser473, and phosphorylation of Thr308 and Ser473 have synergistic effects on activity (Alessi *et al* (1996) *EMBO J.* 15, 6541-6551). An important finding made in the present study is that PKB α does not become phosphorylated at Ser473 when cotransfected with PDK1, and nor does transfection with PDK1 affect the level of Ser473 phosphorylation attained after stimulation with IGF1. These experiments demonstrate that the protein kinase which phosphorylates PKB α at Ser473 is not activated by PDK1, and that phosphorylation of PKB α at Thr308 in a cellular context does not cause PKB α to autophosphorylate at Ser473. Like the phosphorylation of Thr308, Ser473 phosphorylation is prevented by incubating cells with wortmannin prior to stimulation with insulin or IGF1. It will be interesting to know whether the Ser473 kinase is activated by PtdIns(3,4,5)P3 or whether the binding of PtdIns(3,4,5)P3 to PKB α alters its conformation to permit phosphorylation by the Ser473 kinase.

PDK1 was found to be homologous to DSTPK61 a *Drosophila* protein kinase which is involved in the regulation of sex differentiation, oogenesis and spermatogenesis of the fruit fly [22]. The identity between PDK1 and DSTPK1 was 54% in the catalytic domain, but even greater (61 % identity) in the putative PH domain (Fig 11), further suggesting an important functional role for the PH domain of PDK1. DSTPK61 expressed in *E. coli* also activated human PKB α and phosphorylated it specifically at Thr308 in a PtdIns(3,4,5)P₃/PtdIns(3,4)P₂-dependent manner (Fig 12). These findings indicate that DSTPK61 is a *Drosophila* homologue of PDK1 and suggest that one of its roles may be to activate the *Drosophila* homologue of PKB (termed DPKB) that was identified several years ago [31,32]. Although it is not yet known what the physiological roles of DSTPK1 are in flies the findings presented in this paper suggest that DPKB may lie downstream of DSTPK in signalling pathways regulating sex differentiation and oogenesis [22]. Since PtdIns 3-kinases are known to play a major role in the activation of PKB in mammalian cells [4-6 and 13] it is likely that a *Drosophila* phosphoinositide 3-kinase (PtdIns 3-kinase) also plays an important role in the activation of DPKB. Again very little is known about the role of PtdIns 3 kinase in flies [33]. Only one study has been published to date to address this issue in which a *Drosophila* PtdIns 3-kinase catalytic subunit termed Dp110 has been overexpressed in the wing and eye imaginal discs of *Drosophila*, in a normal, a constitutively active and a dominant negative form [34]. The results of these studies showed that PtdIns 3-kinase is likely to play a role in regulating cell growth [34]. Interestingly, loss of function mutations in the *Drosophila* homologue of the insulin receptor, Inr also inhibited cell growth in imaginal discs and resulted in much smaller than wild type flies [35]. Taken together these results suggest that *Drosophila* PtdIns 3-kinases might be important targets of the insulin receptor, perhaps by acting through DPKB may regulate cell growth.

The DSTPK61 gene is differentially spliced in male and female *Drosophila* [22]. This generates many different transcripts that all have the same open reading frame, and therefore produce the same protein, but differ significantly in their 5' and 3' untranslated regions. This is likely to result in different levels of expression of the DSTPK61 protein in male and female *Drosophila* [22]. In view of the role of PKB in regulating apoptosis in mammalian cells (see introduction) it is possible that the function of DSTPK61 during oogenesis, spermatogenesis and in female adults in relation to sex-determinants might be related to cell survival or cell death decisions. One could imagine the low level of the non-sex specific transcript of DSTPK61 being responsible for general growth function consistent with the role of overexpression of Dp110 in cells [34] and the specifically spliced forms seen in female carcasses and ovaries and male testes being controlled translationally leading to the generation of high levels of protein at specific times. It should be remembered that the extent and duration of activation of the MAP kinase cascade is critical in determining whether a signal induces the proliferation of PC12 cells or their differentiation to a sympathetic neurone like phenotype [36,37]. Similarly, different levels of expression of DSTPK61 are likely to affect the extent and duration of activation of PKB *in vivo* and hence may determine the precise function of this kinase cascade. There are two signalling pathways involved in sex differentiation where such a situation may operate. The first involves the development of a specific adult male muscle in the abdomen required for holding the female fly during mating [38]. This muscle only develops in males in response to an unknown signal generated by adjacent nerve cells. It is likely that a signalling pathway which causes muscle cells in that region of the female fly to apoptose, whereas in male flies the growth of these muscle cells is promoted [38]. The second pathway involves an unknown signalling mechanism that operates between the somatic cells and germ cells in the

male and female gonads in which a signal derived from the somatic cells initiates a signalling pathway that promotes cell survival of germ-line cells and induces these to differentiate into sperm and oocytes [39,40]. The mediators of both of these signalling pathways are at present unknown but, in light of the findings presented in this paper it is possible that a signalling pathway may operate that the unknown signals lead to the activation of PtdIns 3-kinase, DSTPK and then DPKB and the role of this pathway could be to mediate cell survival by inhibiting apoptosis. To understand the function of these signalling pathways future work will have to concentrate in obtaining mutants of the *Drosophila* DSTPK61, PKB and PtdIns 3-kinase genes, as well as identifying the specific signals that switch on these pathways. It would be of considerable interest to compare the effects of expressing constitutively active and dominant negative forms of Inr, Dp110, DSTPK61 and DPKB during oogenesis, spermatogenesis and sex-specific muscle differentiation.

Summary

Background. The activation of protein kinase B (PKB, also known as c-Akt) is triggered within minutes of cells being stimulated with insulin or growth factors and results from its phosphorylation at Thr308 and Ser473. The activation of PKB and its phosphorylation at both residues are prevented by inhibitors of phosphoinositide 3-kinase, and we recently identified and purified a protein kinase that phosphorylates PKB at Thr308. This enzyme only phosphorylated PKB in the presence of lipid vesicles containing phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] or phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P₂] and was therefore termed PtdIns(3,4,5)P₃-dependent protein kinase-1 (PDK1).

Results. We have cloned and sequenced human PDK1. The 556 residue, 63.1 kDa enzyme comprises a catalytic domain (residues 83-344) that is most similar to the subfamily of protein kinases that include PKA, PKB and PKC, and possesses a C-terminal pleckstri homology (PH) domain (residues 450-550). The gene encoding PDK1 is located on human chromosome 16p13.3 and is ubiquitously expressed in human tissues. Human PDK1 is highly homologous to the *Drosophila* protein kinase DSTPK61, which has been implicated in the regulation of sex differentiation, oogenesis and spermatogenesis in the fly, with 54% identity between the catalytic domains and 61% identity between the PH domains. GST-PDK1 expressed in 293 cells and GST-DSTPK61 expressed in *E. coli* were purified to homogeneity by chromatography on glutathione-Sepharose and their properties were found to be indistinguishable from PDK1 isolated from rabbit skeletal muscle. In the presence of lipid vesicles containing PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂, but not PtdIns(4,5)P₂ or PtdIns(3)P, GST-PDK1 and GST-DSTPK61 both phosphorylated GST-PKBα stoichiometrically at Thr308 and increased its activity 35-fold *in vitro*. Myc-epitope-tagged human PDK1 also activated HA-tagged PKBα 20-fold in cotransfection experiments in 293 cells, and potentiated the IGF1-induced phosphorylation of HA-tagged PKBα at Thr308.

Overexpression of PDK1 in unstimulated 293 cells resulted in a 20-fold activation of PKBα, and potentiated the IGF1-induced phosphorylation of PKBα at Thr308. Experiments in which the PH domains of either PDK1 or PKBα were deleted indicated that the binding of PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ to PKBα is required for phosphorylation and activation by PDK1. IGF1 stimulation of 293 cells did not affect the activity or phosphorylation state of PDK1.

Conclusions. PDK1 is likely to mediate the activation of PKB by insulin or growth factors. DSTPK61 is a *Drosophila* homologue of PDK1, suggesting that PtdIns(3,4,5)P₃ and/or PtdIns(3,4)P₂ are likely to play as yet unidentified roles in sex differentiation in this organism. The effect of PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ in the activation of PKB α is at least partly substrate directed.

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10 Example 6: Assay for compounds which activate PDK1

An assay is set up with PDK1, GST-PKB α and the PKB α substrate, RPRAATF, (as described in Example 1) but with no 3-phosphoinositide. No activation of PKB α is observed. Compounds are tested in the assay
 15 and those that give rise to activation of PKB α *via* PDK1 are selected for further study. Phosphatidylinositol-3,4,5-trisphosphate is used as a positive control.

Example 7: Assay for compounds which inactivate PDK1

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An assay is set up as above except that it includes phosphatidyl-3,4,5-trisphosphate and therefore PDK1 is active with respect to phosphorylating PKB α .

25 Compounds are tested in the assay and those that lead to inactivation of PKB α *via* PDK1 are selected for further study.

Example 8

30 The assay is the same as Example 6 except that PKB α is replaced by

PKB β .

Example 9

- 5 The assay is the same as Example 6 except that PKB α is replaced by PKB γ .

Example 10

- 10 The assay is the same as Example 7 except that PKB α is replaced by PKB β .

Example 11

- 15 The assay is the same as Example 7 except that PKB α is replaced by PKB γ .

Example 12: Assay for compounds which compete for phosphatidylinositol-3,4,5-trisphosphate-binding of PDK1

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PDK1 is incubated with radiolabelled phosphatidylinositol-3,4,5-trisphosphate in the presence of a test compound. Binding of the phospholipid to PDK1 in the presence of the test compound is compared to the binding in its absence.

25

Compounds which reduce or enhance the binding are selected for further study.

The assay may also be carried out with non-radiolabelled PtdIns(3,4,5)P₃.

30

Example 13: Assay for compounds which activate PDK1

An assay is set up with PDK1 and p70 S6 kinase. No 3-phosphoinositide need be present. Compounds are tested in the assay and those that
5 activate PDK1 (and lead to increased phosphorylation of p70 S6 kinase) are selected.

Example 14: Assay for compounds which activate PDK1

10 An assay is set up with PDK1 and a PKB α lacking a functional PH domain. No 3-phosphoinositide need be present. Compounds are tested and those that activate PDK1 (and lead to increased phosphorylation of PKB α lacking a functional PH domain) are selected.

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SEQUENCE LISTING

1) GENERAL INFORMATION:

- (i) APPLICANT: Alessi, Dario R
- (ii) TITLE OF INVENTION: ENZYME
- (iii) NUMBER OF SEQUENCES: 35
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/943,667
 - (B) FILING DATE: 03-OCT-1997
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 - (B) FILING DATE: 15-AUG-1997
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(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 556 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met	Ala	Arg	Thr	Thr	Ser	Gln	Leu	Tyr	Asp	Ala	Val	Pro	Ile	Gln	Ser	
1				5					10					15		
Ser	Val	Val	Leu	Cys	Ser	Cys	Pro	Ser	Pro	Ser	Met	Val	Arg	Thr	Gln	
			20					25					30			
Thr	Glu	Ser	Ser	Thr	Pro	Pro	Gly	Ile	Pro	Gly	Gly	Ser	Arg	Gln	Gly	
		35					40					45				
Pro	Ala	Met	Asp	Gly	Thr	Ala	Ala	Glu	Pro	Arg	Pro	Gly	Ala	Gly	Ser	
	50					55					60					
Leu	Gln	His	Ala	Gln	Pro	Pro	Pro	Gln	Pro	Arg	Lys	Lys	Arg	Pro	Glu	
65					70					75					80	
Asp	Phe	Lys	Phe	Gly	Lys	Ile	Leu	Gly	Glu	Gly	Ser	Phe	Ser	Thr	Val	
				85					90					95		
Val	Leu	Ala	Arg	Glu	Leu	Ala	Thr	Ser	Arg	Glu	Tyr	Ala	Ile	Lys	Ile	
			100					105					110			
Leu	Glu	Lys	Arg	His	Ile	Ile	Lys	Glu	Asn	Lys	Val	Pro	Tyr	Val	Thr	
		115					120					125				
Arg	Glu	Arg	Asp	Val	Met	Ser	Arg	Leu	Asp	His	Pro	Phe	Phe	Val	Lys	
	130					135					140					
Leu	Tyr	Phe	Thr	Phe	Gln	Asp	Asp	Glu	Lys	Leu	Tyr	Phe	Gly	Leu	Ser	
145					150					155					160	
Tyr	Ala	Lys	Asn	Gly	Glu	Leu	Leu	Lys	Tyr	Ile	Arg	Lys	Ile	Gly	Ser	
				165					170					175		
Phe	Asp	Glu	Thr	Cys	Thr	Arg	Phe	Tyr	Thr	Ala	Glu	Ile	Val	Ser	Ala	
			180					185					190			
Leu	Glu	Tyr	Leu	His	Gly	Lys	Gly	Ile	Ile	His	Arg	Asp	Leu	Lys	Pro	

195					200					205					
Glu	Asn	Ile	Leu	Leu	Asn	Glu	Asp	Met	His	Ile	Gln	Ile	Thr	Asp	Phe
210						215					220				
Gly	Thr	Ala	Lys	Val	Leu	Ser	Pro	Glu	Ser	Lys	Gln	Ala	Arg	Ala	Asn
225					230					235					240
Ser	Phe	Val	Gly	Thr	Ala	Gln	Tyr	Val	Ser	Pro	Glu	Leu	Leu	Thr	Glu
				245					250					255	
Lys	Ser	Ala	Cys	Lys	Ser	Ser	Asp	Leu	Trp	Ala	Leu	Gly	Cys	Ile	Ile
			260					265					270		
Tyr	Gln	Leu	Val	Ala	Gly	Leu	Pro	Pro	Phe	Arg	Ala	Gly	Asn	Glu	Tyr
		275					280					285			
Leu	Ile	Phe	Gln	Lys	Ile	Ile	Lys	Leu	Glu	Tyr	Asp	Phe	Pro	Glu	Lys
	290					295					300				
Phe	Phe	Pro	Lys	Ala	Arg	Asp	Leu	Val	Glu	Lys	Leu	Leu	Val	Leu	Asp
305					310					315					320
Ala	Thr	Lys	Arg	Leu	Gly	Cys	Glu	Glu	Met	Glu	Gly	Tyr	Gly	Pro	Leu
				325					330					335	
Lys	Ala	His	Pro	Phe	Phe	Glu	Ser	Val	Thr	Trp	Glu	Asn	Leu	His	Gln
			340					345					350		
Gln	Thr	Pro	Pro	Lys	Leu	Thr	Ala	Tyr	Leu	Pro	Ala	Met	Ser	Glu	Asp
		355					360					365			
Asp	Glu	Asp	Cys	Tyr	Gly	Asn	Tyr	Asp	Asn	Leu	Leu	Ser	Gln	Phe	Gly
	370					375					380				
Cys	Met	Gln	Val	Ser	Ser	Ser	Ser	Ser	Ser	His	Ser	Leu	Ser	Ala	Ser
385					390					395					400
Asp	Thr	Gly	Leu	Pro	Gln	Arg	Ser	Gly	Ser	Asn	Ile	Glu	Gln	Tyr	Ile
				405					410					415	
His	Asp	Leu	Asp	Ser	Asn	Ser	Phe	Glu	Leu	Asp	Leu	Gln	Phe	Ser	Glu
		420						425					430		
Asp	Glu	Lys	Arg	Leu	Leu	Leu	Glu	Lys	Gln	Ala	Gly	Gly	Asn	Pro	Trp
		435					440					445			
His	Gln	Phe	Val	Glu	Asn	Asn	Leu	Ile	Leu	Lys	Met	Gly	Pro	Val	Asp
	450					455					460				
Lys	Arg	Lys	Gly	Leu	Phe	Ala	Arg	Arg	Arg	Gln	Leu	Leu	Leu	Thr	Glu
465					470					475					480
Gly	Pro	His	Leu	Tyr	Tyr	Val	Asp	Pro	Val	Asn	Lys	Val	Leu	Lys	Gly

	485		490		495
Glu Ile Pro Trp Ser Gln Glu Leu Arg Pro Glu Ala Lys Asn Phe Lys	500	505	510		
Thr Phe Phe Val His Thr Pro Asn Arg Thr Tyr Tyr Leu Met Asp Pro	515	520	525		
Ser Gly Asn Ala His Lys Trp Cys Arg Lys Ile Gln Glu Val Trp Arg	530	535	540		
Gln Arg Tyr Gln Ser His Pro Asp Ala Ala Val Gln	545	550	555		

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1670 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGGCCAGGA CCACCAGCCA GCTGTATGAC GCCGTGCCCA TCCAGTCCAG CGTGGTGTTA	60
TGTTCTTGCC CATCCCCATC AATGGTGAGG ACCCAGACTG AGTCCAGCAC GCCCCCTGGC	120
ATTCCTGGTG GCAGCAGGCA GGGCCCCGCC ATGGACGGCA CTGCAGCCGA GCCTCGGCCC	180
GGCGCCGGCT CCCTGCAGCA TCCCAGCCTC CGCCGCAGCC TCGGAAGAAG CGGCCTGAGG	240
ACTTCAAGTT TGGGAAAATC CTTGGGGAAG GCTCTTTTTC CACGGTTGTC CTGGCTCGAG	300
AACTGGCAAC CTCCAGAGAA TATGCGATTA AAATTCTGGA GAAGCGACAT ATCATAAAAG	360
AGAACAAGGT CCCCTATGTA ACCAGAGAGC GGGATGTCAT GTCGCGCCTG GATCACCCCT	420
TCTTTGTTAA GCTTTACTTC ACATTTCAGG ACGACGAGAA GCTGTATTTT GGCCTTAGTT	480
ATGCCAAAAA TGGAGAACTA CTTAAATATA TTCGCAAAAT CGGTTTCATTC GATGAGACCT	540
GTACCCGATT TTACACGGCT GAGATCGTGT CTGCTTTAGA GTAATTGCAC GGCAAGGGCA	600

TCATTCACAG	GGACCTTAAA	CCGGAAAACA	TTTTGT TAAA	TGAAGATATG	CACATCCAGA	660
TCACAGATTT	TGGAACAGCA	AAAGTCTTAT	CCCCAGAGAG	CAAACAAGCC	AGGGCCAACT	720
CATTCGTGGG	AACAGCGCAG	TACGTTTCTC	CAGAGCTGCT	CACGGAGAAG	TCCGCCTGTA	780
AGAGTTCAGA	CCTTTGGGCT	CTTGGATGCA	TAATATACCA	GCTTGTGGCA	GGACTCCCAC	840
CATTCCGAGC	TGGAAACGAG	TATCTTATAT	TTCAGAAGAT	CATTAAGTTG	GAATATGACT	900
TTCCAGAAAA	ATTCTTCCCT	AAGGCAAGAG	ACCTCGTGGA	GAAACTTTTG	GTTTTAGATG	960
CCACAAAGCG	GTTAGGCTGT	GAGGAAATGG	AAGGATACGG	ACCTCTTAAA	GCACACCCGT	1020
TCTTCGAGTC	CGTCACGTGG	GAGAACCTGC	ACCAGCAGAC	GCCTCCGAAG	CTCACCGCTT	1080
ACCTGCCGGC	TATGTCGGAA	GACGACGAGG	ACTGCTATGG	CAATTATGAC	AATCTCCTGA	1140
GCCAGTTTGG	CTGCATGCAG	GTGTCTTCGT	CCTCCTCCTC	ACACTCCCTG	TCAGCCTCCG	1200
ACACGGGCCT	GCCCCAGAGG	TCAGGCAGCA	ACATAGAGCA	GTACATTCAC	GATCTGGACT	1260
CGAACTCCTT	TGAACTGGAC	TTACAGTTTT	CCGAAGATGA	GAAGAGGTTG	TTGTTGGAGA	1320
AGCAGGCTGG	CGGAAACCCT	TGGCACCAGT	TTGTAGAAAA	TAATTTAATA	CTAAAGATGG	1380
GCCCAGTGGA	TAAGCGGAAG	GGTTTATTTG	CAAGACGACG	ACAGCTGTTG	CTCACAGAAG	1440
GACCACATTT	ATATTATGTG	GATCCTGTCA	ACAAAGTTCT	GAAAGGTGAA	ATTCCTTGGT	1500
CACAAGAACT	TCGACCAGAG	GCCAAGAATT	TTAAAACTTT	CTTTGTCCAC	ACGCCTAACA	1560
GGACGTATTA	TCTGATGGAC	CCCAGCGGGA	ACGCACACAA	GTGGTGCAGG	AAGATCCAGG	1620
AGGTTTGGAG	GCAGCGATAC	CAGAGCCACC	CGGACGCCGC	TGTGCAGTGA		1670

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Asn Ser Phe Val Gly Thr Ala Gln Tyr Val Ser Pro Glu Leu Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Gly Asn Glu Tyr Leu Ile Phe Gln Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Leu Asp His Pro Phe Phe Val Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Arg	Gln	Arg	Tyr	Gln	Ser	His	Pro	Asp	Ala	Ala	Val	Gln
1				5							10	

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Leu	Ser	Pro	Glu	Ser	Lys	Gln	Ala	Arg	Ala	Asn	Ser
1				5						10	

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 11 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Thr	Ala	Ser	Gly	Val	Ala	Glu	Thr	Thr	Asn	Cys
1				5					10	

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Arg	Pro	Arg	Ala	Ala	Thr	Phe
1				5		

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Thr Thr Tyr Ala Asp Phe Ile Ala Ser Gly Arg Thr Gly Arg Arg Asn
1 5 10 15

Ala Ile His Asp
20

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ala His Pro Phe Phe Glu Ser Val Thr Trp Glu Asn Leu His Gln Gln
1 5 10 15

Thr Pro Pro Lys
20

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ser Gly Ser Asn Ile Glu Gln Tyr Ile His Asp Leu Asp Ser Asn Ser
1 5 10 15

Phe Glu Leu Asp Leu
20

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Gln	Ala	Gly	Gly	Asn	Pro	Trp	His	Gln	Phe	Val	Glu	Asn	Asn	Leu	Ile
1				5					10					15	
Leu Lys															

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Gln	Leu	Leu	Leu	Thr	Glu	Gly	Pro	His	Leu	Tyr	Tyr	Val	Asp	Pro	Val
1				5					10					15	
Asn Lys															

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TGAGCCAGT TTGGCTGC

18

2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

ACGTCCTGTT AGGCGTGTGG

20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TTGTTKGGNA CNGCNCARTA YGT

23

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ATTNACRAARA ANGGRTGRTC

20

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

AAYGARTAYY TNATHTTYCA RAA

23

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 73 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TCGGAGATCT GCCACCATGG AGCAGAAGCT GATCTCTGAA GAGGACTTGG ACGGCACTGC 60

AGCCGAGCCT CGG 73

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TCGGGGTACC TCACTGCACA GCGGCGTCCG GGTG 34

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TCGGAGATCT GCCACCATGG AGCAGAAGCT GATCTCTGAA GAGGACTTGG CCAGGACCAC 60

CAGCCAGCTG TATGACG 77

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

3CGGGGTACC TCAGTGCCAA GGGTTTCCGC CAGCCTGCTT

40

- (2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CGGGATCCAT GGACTTCCGG TCGGGCTCA

29

- (2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 419 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GAGTCCAGC ACGCCCCCTG NATTTCCTGG TGGCAGCAGG CANGGGCCCCG CATGGACGGC	60
CTGCAGCCG AGCCTCGNCC AGGGCCGGCA TCCCATGCAG CATGCCAGCC TCCGCCGCAG	120
TCGGAAGAA GCGGCCTGAG GACTTCAAGT TTGGGAAAAT CTTGGGGAAG GCTCTTTTNC	180
.CGGTTGTCT GGCTCGAGAA CTGGCAACCT CCAGAGAATA TGCGATTAAA ATNCTGGAGA	240
.GCGACATAT CATAAAAGAG AACAAGGTCC CTATGTAACC AGAGANTGGG ATGTCATGTC	300
ICGCCTGGAT CACCCCTTCT TTGTTAAGCT TTA CTTCACA TTTCAGGACG ACGAGAAGNT	360
ITATTTCCGC CTTAGTTATG CNAAAAATTG GAGAACTACT TAAATATATT CGCAAAATC	419

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 548 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TTAAATAAAA ATGCTGCAAG GTTTCGCCT CTGCGTTCCC CTTGTGATGG CTGGCAGGTG	60
GTCTGGAAGC GTCCCGGATG GCGGCCAAGC CGGCGAGGGG CAGGTGTCCT GGCAGCGAAG	120
GGCAGCCCCG CCGCACNCGA TCACTGCACA GCGGCGTCCG GGTGGCTCTG GTATCGCTGC	180
CTCCAAACCT CCTGGATCTT CCTGCACCAC TTGTGTGCGT TCCCGCTGGG GTCCATCAGA	240
TAATACGTCC TGTTAGGCGT GTGGACAAAG AAAGTTTTAA AATTCTTGGC CTCTGGTCNA	300
AGTTCTTGTG ACCAAGGAAT TTCACCTTTC AGAACTTTGT TGACAGGATC CACATAATAT	360
AAATGTGGTC CTCTGTGAGC AACAGCTGTC GTCGTCTTGC AAATAAACCC TTCCGCTTAT	420
CCACTGGGCC CATCTTTAGT ATTAAATTAT TTTCTACAAA CTGGTGNCAA GGGTTTCCGC	480

VAGCCTGGCT TCTCCAACAA CAANCTCTTC TCATCTTCGG AAAACTGTAA GTCCAGTTCA 540

VAGGAGTT 548

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 752 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Drosophila

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Met	Ala	Lys	Glu	Lys	Ala	Ser	Ala	Thr	Val	Ser	Leu	Gly	Glu	Ser	Asn	
1				5					10					15		
Phe	Arg	Asp	Ile	Asn	Leu	Lys	Asp	Leu	Ala	Val	Val	Val	Glu	Ala	Ala	
			20					25					30			
Ser	Arg	Leu	His	His	Gln	Gln	Asn	Val	Cys	Gly	Cys	Gly	Ala	Val	Ser	
		35					40					45				
Ser	Thr	Glu	Asn	Asn	Asn	Asn	Ser	Arg	Tyr	Gly	Ser	Ser	Lys	Tyr	Leu	
	50					55					60					
Thr	Asn	Gly	His	Thr	Ser	Pro	Leu	Ala	Ala	Ala	Val	Ala	Ser	Asn	Ser	
65					70				75					80		
Ser	Ser	Val	Ala	Thr	Thr	Pro	His	Cys	Arg	Met	Leu	His	Asn	Cys	Ser	
				85					90					95		
Leu	Gln	Gln	Tyr	Gln	Asn	Asp	Ile	Arg	Gln	Gln	Thr	Glu	Ile	Leu	Asp	
			100					105					110			
Met	Leu	Arg	His	Glu	His	Gln	Gln	Gly	Tyr	Gln	Ser	Gln	Gln	Gln	Gln	
		115					120					125				
Gln	Gln	Pro	Gln	Gln	Gln	Gln	Glu	Gln	Gln	Gln	Gln	Gln	Glu	Gln	Ser	
		130					135					140				
Gln	Gln	Gln	Gln	Gln	Leu	Gln	Asn	Pro	Ala	Pro	Arg	Arg	Ser	Pro	Asn	
145					150					155					160	

Asp Phe Ile Phe Gly Arg Tyr Ile Gly Glu Gly Ser Tyr Ser Ile Val
165 170 175

Tyr Leu Ala Val Asp Ile His Ser Arg Arg Glu Tyr Ala Ile Lys Val
180 185 190

Cys Glu Lys Arg Leu Ile Leu Arg Glu Arg Lys Gln Asp Tyr Ile Lys
195 200 205

Arg Glu Arg Glu Val Met His Gln Met Thr Asn Val Pro Gly Phe Val
210 215 220

Asn Leu Ser Cys Thr Phe Gln Asp Ala Arg Ser Leu Tyr Phe Val Met
225 230 235 240

Thr Tyr Ala Arg Lys Gly Asp Met Leu Pro Tyr Ile Asn Arg Val Gly
245 250 255

Ser Phe Asp Val Ala Cys Thr Arg His Tyr Ala Ala Glu Leu Leu Leu
260 265 270

Ala Cys Glu His Met His Arg Arg Asn Val Val His Arg Asp Leu Lys
275 280 285

Pro Glu Asn Ile Leu Leu Asp Glu Asp Met His Thr Leu Ile Ala Asp
290 295 300

Phe Gly Ser Ala Lys Val Met Thr Ala His Glu Arg Ala Leu Ala Thr
305 310 315 320

Glu His Cys Ser Glu Gln Arg Arg Ser Asn Ser Asp Glu Asp Asp Glu
325 330 335

Asp Ser Asp Arg Leu Glu Asn Glu Asp Glu Asp Phe Tyr Asp Arg Asp
340 345 350

Ser Glu Glu Leu Asp Asp Arg Asp Asp Glu Gln Gln Gln Glu Glu Met
355 360 365

Asp Ser Pro Arg His Arg Gln Arg Arg Tyr Asn Arg His Arg Lys Ala
370 375 380

Ser Phe Val Gly Thr Ala Gln Tyr Val Ser Pro Glu Val Leu Gln Asn
385 390 395 400

Gly Pro Ile Thr Pro Ala Ala Asp Leu Trp Ala Leu Gly Cys Ile Val
405 410 415

Tyr Gln Met Ile Ala Gly Leu Pro Pro Phe Arg Gly Ser Asn Asp Tyr
420 425 430

Val Ile Phe Lys Glu Ile Leu Asp Cys Ala Val Asp Phe Pro Gln Gly
435 440 445

Phe	Asp	Lys	Asp	Ala	Glu	Asp	Leu	Val	Arg	Lys	Leu	Leu	Arg	Val	Asp
450						455					460				
Pro	Arg	Asp	Arg	Leu	Gly	Ala	Gln	Asp	Glu	Phe	Gly	Tyr	Tyr	Glu	Ser
465					470					475					480
Ile	Arg	Ala	His	Pro	Phe	Phe	Ala	Gly	Ile	Asp	Trp	Gln	Thr	Leu	Arg
				485					490					495	
Gln	Gln	Thr	Pro	Pro	Pro	Ile	Tyr	Pro	Tyr	Leu	Pro	Gly	Val	Ser	Gln
			500					505					510		
Asp	Glu	Asp	Phe	Arg	Ser	Ser	Tyr	Thr	Val	Pro	Gly	Asp	Leu	Glu	Pro
		515					520					525			
Gly	Leu	Asp	Glu	Arg	Gln	Ile	Ser	Arg	Leu	Leu	Ser	Ala	Glu	Leu	Gly
	530					535					540				
Val	Gly	Ser	Ser	Val	Ala	Met	Pro	Val	Lys	Arg	Ser	Thr	Ala	Lys	Asn
545					550					555					560
Ser	Phe	Asp	Leu	Asn	Asp	Ala	Glu	Lys	Leu	Gln	Arg	Leu	Glu	Gln	Gln
				565					570					575	
Lys	Thr	Asp	Lys	Trp	His	Val	Phe	Ala	Asp	Gly	Glu	Val	Ile	Leu	Lys
			580					585					590		
Lys	Gly	Phe	Val	Asn	Lys	Arg	Lys	Gly	Leu	Phe	Ala	Arg	Lys	Arg	Met
		595					600					605			
Leu	Leu	Leu	Thr	Thr	Gly	Pro	Arg	Leu	Ile	Tyr	Ile	Asp	Pro	Val	Gln
	610					615					620				
Met	Ile	Lys	Lys	Gly	Glu	Ile	Pro	Trp	Ser	Pro	Asp	Leu	Arg	Ala	Glu
625					630					635					640
Tyr	Lys	Asn	Phe	Lys	Ile	Phe	Phe	Val	His	Thr	Pro	Asn	Arg	Thr	Tyr
				645					650					655	
Tyr	Leu	Asp	Asp	Pro	Glu	Gly	Tyr	Ala	Ile	His	Trp	Ser	Glu	Ala	Ile
			660					665					670		
Glu	Asn	Met	Arg	Lys	Leu	Ala	Tyr	Gly	Asp	Pro	Ser	Ser	Thr	Ser	Ala
		675					680					685			
Val	Ser	Cys	Ser	Ser	Gly	Ser	Ser	Asn	Ser	Leu	Ala	Val	Ile	Ser	Asn
	690					695					700				
Ser	Ser	Ala	Ala	Ser	Ser	Ser	Asn	Ser	Pro	Thr	Val	Lys	Arg	Ser	Ser
705					710					715					720
Pro	Val	Asn	Ala	Pro	Gln	Ala	Ser	Thr	Ala	Ser	Asp	Asn	Arg	Thr	Leu
				725					730					735	

Gly Ser Thr Arg Thr Gly Thr Ser Pro Ser Lys Lys Thr Ala Ser Lys
740 745 750

(2) INFORMATION FOR SEQ ID NO: 30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 92 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Lys	Met	Gly	Pro	Val	Asp	Lys	Arg	Lys	Gly	Leu	Phe	Ala	Arg	Arg	Arg	1	5	10	15
Gln	Leu	Leu	Leu	Thr	Glu	Gly	Pro	His	Leu	Tyr	Tyr	Val	Asp	Pro	Val	20	25	30	
Asn	Lys	Val	Leu	Lys	Gly	Glu	Ile	Pro	Trp	Ser	Gln	Glu	Leu	Arg	Pro	35	40	45	
Glu	Ala	Lys	Asn	Phe	Lys	Thr	Phe	Phe	Val	His	Thr	Pro	Asn	Arg	Thr	50	55	60	
Tyr	Tyr	Leu	Met	Asp	Pro	Ser	Gly	Asn	Ala	His	Lys	Trp	Cys	Arg	Lys	65	70	75	80
Ile	Gln	Glu	Val	Trp	Arg	Gln	Arg	Tyr	Gln	Ser	His	85	90						

(2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 92 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Lys	Lys	Gly	Phe	Val	Asn	Lys	Arg	Lys	Gly	Leu	Phe	Ala	Arg	Lys	Arg	1	5	10	15
Met	Leu	Leu	Leu	Thr	Thr	Gly	Pro	Arg	Leu	Ile	Tyr	Ile	Asp	Pro	Val	20	25	30	
Gln	Met	Ile	Lys	Lys	Gly	Glu	Ile	Pro	Trp	Ser	Pro	Asp	Leu	Arg	Ala	35	40	45	
Glu	Tyr	Lys	Asn	Phe	Lys	Ile	Phe	Phe	Val	His	Thr	Pro	Asn	Arg	Thr	50	55	60	
Tyr	Tyr	Leu	Asp	Asp	Pro	Glu	Gly	Tyr	Ala	Ile	His	Trp	Ser	Glu	Ala	65	70	75	80
Ile	Glu	Asn	Met	Arg	Lys	Leu	Ala	Tyr	Gly	Asp	Pro	85	90						

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 101 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Arg	Glu	Gly	Tyr	Leu	Val	Lys	Lys	Gly	Ser	Val	Phe	Asn	Thr	Trp	Lys	1	5	10	15
Pro	Met	Trp	Val	Val	Leu	Leu	Glu	Asp	Gly	Ile	Glu	Phe	Tyr	Lys	Lys	20	25	30	
Lys	Ser	Asp	Asn	Ser	Pro	Lys	Gly	Met	Ile	Pro	Leu	Lys	Gly	Ser	Thr	35	40	45	

Leu Thr Ser Pro Cys Gln Asp Phe Gly Lys Arg Met Phe Val Phe Lys
 50 55 60

Ile Thr Thr Thr Lys Gln Gln Asp His Phe Phe Gln Ala Ala Phe Leu
 65 70 75 80

Glu Glu Arg Asp Ala Trp Val Arg Asp Ile Asn Lys Ala Ile Lys Cys
 85 90 95

Ile Glu Gly Leu Glu
 100

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Met Glu Gly Phe Leu Asn Arg Lys His Glu Trp Glu Ala His Asn Lys
 1 5 10 15

Lys Ala Ser Ser Arg Ser Trp His Asn Val Tyr Gly Val Ile Asn Asn
 20 25 30

Gln Glu Met Gly Phe Tyr Lys Asp Ala Lys Ser Ala Ala Ser Gly Ile
 35 40 45

Pro Tyr His Ser Glu Val Pro Val Ser Leu Lys Glu Ala Ile Cys Glu
 50 55 60

Val Ala Leu Asp Tyr Lys Lys Lys Lys His Val Phe Lys Leu Arg Leu
 65 70 75 80

Ser Asp Gly Asn Glu Tyr Leu Phe Gln Ala Lys Asp Asp Glu Glu Met
 85 90 95

Asn Thr Trp Ile Gln Ala Ile Ser Ser Ala Ile Ser Ser Asp Lys His
 100 105 110

2) INFORMATION FOR SEQ ID NO: 34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 109 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Arg	Lys	Gly	Trp	Leu	Thr	Ile	Asn	Asn	Ile	Gly	Ile	Met	Lys	Gly	Gly	
1				5					10					15		
Ser	Lys	Glu	Tyr	Trp	Phe	Val	Leu	Thr	Ala	Glu	Asn	Leu	Ser	Trp	Tyr	
			20					25					30			
Lys	Asp	Asp	Glu	Glu	Lys	Glu	Lys	Lys	Tyr	Met	Leu	Ser	Val	Asp	Asn	
		35					40					45				
Leu	Lys	Leu	Arg	Asp	Val	Glu	Lys	Gly	Phe	Met	Ser	Ser	Lys	His	Ile	
		50				55					60					
Phe	Ala	Leu	Phe	Asn	Thr	Glu	Gln	Arg	Asn	Val	Tyr	Lys	Asp	Tyr	Arg	
65					70					75					80	
Gln	Leu	Glu	Leu	Ala	Cys	Glu	Thr	Gln	Glu	Glu	Val	Asp	Ser	Trp	Lys	
				85					90					95		
Ala	Ser	Phe	Leu	Arg	Ala	Gly	Val	Tyr	Pro	Glu	Arg	Val				
			100					105								

(2) INFORMATION FOR SEQ ID NO: 35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 114 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

[illegible]

1. A substantially pure human or rabbit 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α , wherein the protein kinase comprises the peptide sequence as shown in SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5.

2. A substantially pure human or rabbit 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α , wherein the protein kinase has a molecular weight of about 67 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and wherein the 3-phosphoinositide-dependent protein kinase comprises the peptide sequence as shown in SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5.

3. A substantially pure 3-phosphoinositide dependent protein kinase having the amino acid sequence as shown in SEQ ID. NO:1 with from 1 to 4 conservative substitutions therein.

4. A substantially pure human or rabbit 3-phosphoinositide-dependent protein kinase according to claim 1 that phosphorylates and activates protein kinase B α .

5. A substantially pure human or rabbit phosphatidylinositol-3,4,5-triphosphate-dependent protein kinase according to Claim 1.

6. A protein kinase according to Claim 1 which activates protein kinase B α in the presence of the D-enantiomer of *sn*-1-stearoyl-2-arachidonyl phosphatidylinositol 3,4,5-trisphosphate but does not activate protein kinase B α in the presence of the L-enantiomer of the said phosphatidylinositol 3,4,5-trisphosphate.

7. A protein kinase according to Claim 1 which is activated by the D-enantiomer of *sn*-1,2-dipalmitoyl phosphatidylinositol 3,4,5-trisphosphate or *sn*-1,2-dipalmitoyl phosphatidylinositol 3,4-bisphosphate but is not activated by the L-enantiomers of the said phosphatidyl inositol phosphates.

8. A protein kinase according to Claim 1 which is not activated by phosphatidylinositol 3,5-bisphosphate or phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 4-phosphate or phosphatidylinositol 3-phosphate or inositol 1,3,4,5-tetrakisphosphate.

9. A protein kinase according to claim 1 isolated from rabbit skeletal muscle.

10. A polynucleotide encoding the protein kinase of claim 1.

11. A polynucleotide according to claim 10 which contains no introns.

12. A vector comprising the polynucleotide of claim 10.

13. A host cell comprising the polynucleotide of claim 10.

14. A host cell comprising the vector of claim 12.

15. A method of making a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α or a fragment or fusion of said protein kinase, the method comprising culturing a host cell of claim 13 which expresses said 3-phosphoinositide-dependent protein kinase and

isolating said 3-phosphoinositide-dependent protein kinase from said host culture.

16. A method of isolating a protein kinase according to claim 1, the method comprising the steps of (a) obtaining material that contains said protein kinase, (b) obtaining cell free extracts from said material which contain said protein kinase, (c) fractionating said cell free extract and (d) selecting a fraction from step (c) which is capable of phosphorylating and activating protein kinase B α in the presence of a 3-phosphoinositide.

17. A human or rabbit 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B α or a fusion protein comprising said protein kinase or a fragment of said fusion protein or a fusion protein comprising said fragment obtainable by the method of Claim 16.

20. A method of identifying a compound that modulates the activity of a protein kinase according to claim 1, the method comprising:

contacting a compound with said protein kinase according to claim 1 or a fusion protein comprising said protein kinase according to claim 1 or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment and

determining whether, in the presence of said compound, phosphorylation and activation of a protein kinase B or phosphorylation of a p70 S6 kinase is changed compared to the phosphorylation and activation of a protein kinase B or phosphorylation of a p70 S6 kinase in the absence of said compound.

21. A method according to Claim 20 wherein the compound decreases the activity of the 3-phosphoinositide-dependent protein kinase.

22. A method according to Claim 20 wherein the compound increases the activity of the 3-phosphoinositide-dependent protein kinase.

23. A method according to Claim 20 wherein the compound competes with a 3-phosphoinositide.

24. A method according to Claim 23 wherein the compound prevents activation of protein kinase B α in the presence of phosphatidylinositol-3,4,5-trisphosphate or phosphatidylinositol-3,4-bisphosphate.

25. A method according to Claim 20 wherein the compound modulates the activity of a human or rabbit 3-phosphoinositide-dependent protein kinase by binding to the protein kinase B α and preventing phosphorylation and activation of protein kinase B α by the 3-phosphoinositide-dependent protein kinase.

26. A method of identifying a compound that mimics the effect of a 3-phosphoinositide on a protein kinase according to claim 1, the method comprising determining whether said compound activates the protein kinase according to claim 1 or a fusion protein comprising said protein kinase according to claim 1 or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment so that it can phosphorylate and activate a protein kinase B or phosphorylate a p70 S6 kinase, the activation by said compound being in the absence of a phosphatidylinositol-3,4,5-trisphosphate or a

phosphatidyl-inositol-3,4-bisphosphate or another 3-phosphoinositide.

27. A method according to Claim 26 wherein the 3-phosphoinositide is phosphatidylinositol-3,4,5-trisphosphate or phosphatidyl-inositol-3,4-bisphosphate.

28. A method of screening for compounds which modulate the activity of a protein kinase according to claim 1, or a fusion protein comprising said protein kinase according to claim 1 or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment, or compounds which modulate their interactions with a 3-phosphoinositide or with protein kinase B, wherein said screening comprises:

contacting a compound with the protein kinase according to claim 1 or a fusion protein comprising said protein kinase according to claim 1 or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment and

selecting compounds which modulate the activity of said protein kinase according to claim 1 or a fusion protein comprising said protein kinase according to claim 1 or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment.

29. A method of activating protein kinase B the method comprising contacting said protein kinase B with a 3-phosphoinositide-dependent protein kinase according to Claim 1.

30. A kit comprising substantially pure human or rabbit 3-phosphoinositide-dependent protein kinase that

phosphorylates and activates protein kinase B α , wherein the protein kinase comprises the peptide sequence as shown in SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO: or a fusion protein comprising said protein kinase or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment and a means for carrying out the method according to Claim 20.

31. A method of activating protein kinase B comprising contacting the protein kinase B with a 3-phosphoinositide-dependent protein kinase as defined in claim 1.

32. A fusion protein comprising the protein kinase according to claim 1 or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment.

33. A substantially pure 3-phosphoinositide-dependent protein kinase wherein the protein kinase comprises amino acids 52-556 of SEQ ID NO:1 or amino acids 52-556 of SEQ ID NO:1 with from 1 to 4 conservative substitutions therein.

34. A fusion protein comprising the protein kinase according to claim 33 or a fragment of said fusion protein having 3-phosphoinositide-dependent protein kinase activity or a fusion protein comprising said fragment.

36. A polypeptide encoding the protein kinase of claim 32.

37. A polypeptide encoding the protein kinase of claim 33.

38. A polypeptide encoding the protein kinase of claim 34.

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